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This supplementary issue  
is dedicated to  
**ALLAN GRANT LOCHHEAD**  
in recognition of his contribution  
to science

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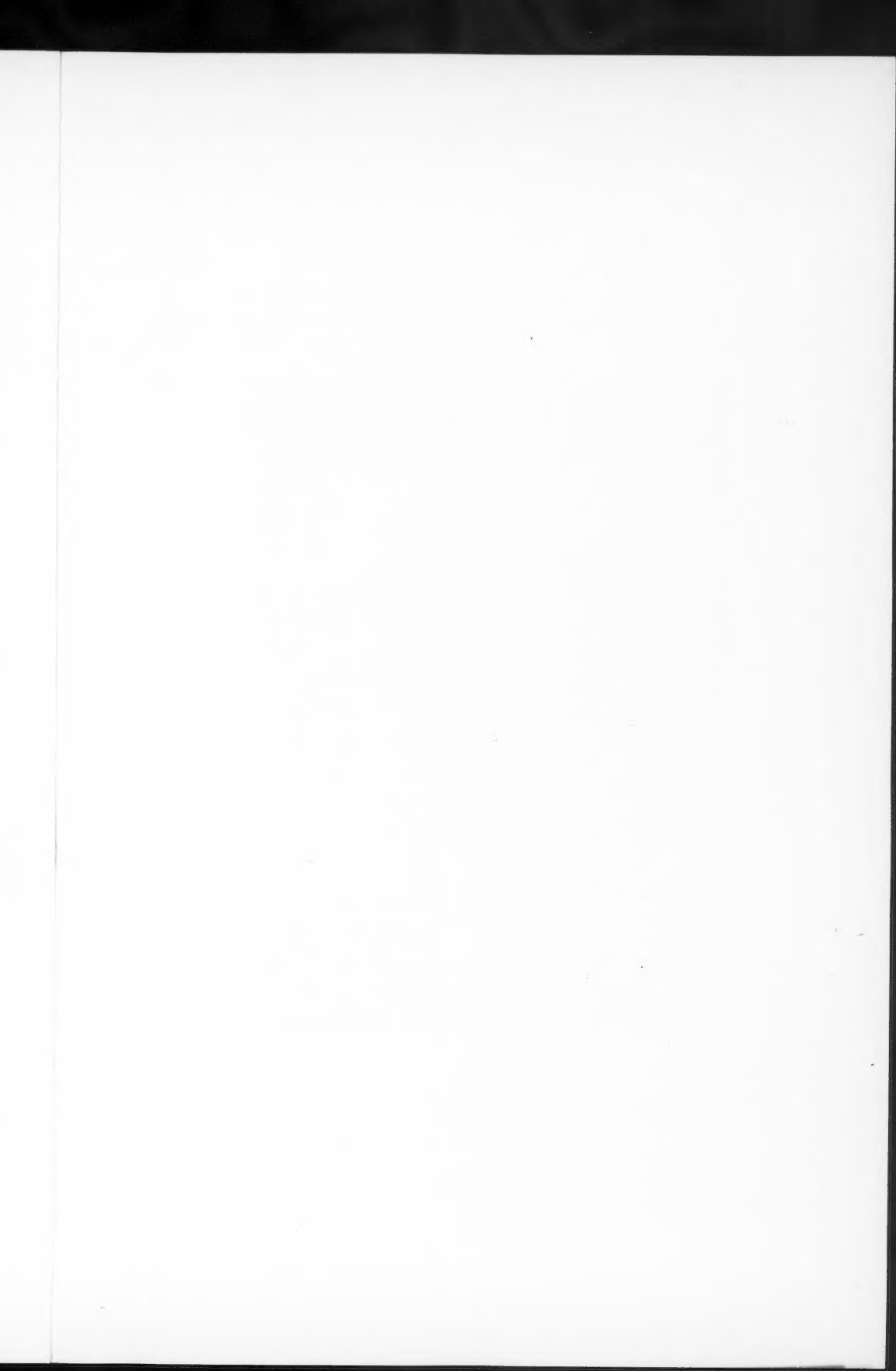
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Chuchuck

To

ALLAN GRANT LOCHHEAD

this issue is dedicated  
by his friends and colleagues as a tribute  
on the occasion of his retirement as Chief  
of the Bacteriology Division,  
Science Service,  
Canada Department of Agriculture



## ALLAN GRANT LOCHHEAD

### An Appreciation<sup>1</sup>

It is appropriate that this brief appreciation of Doctor Lochhead and his work should be devoted mainly to his scientific research. However, readers of this Journal, especially those fortunate enough to know him personally, will be interested in some aspects of his private life in addition to his researches and the inescapable biographical statistics. The fact that the little time he felt able to spare from his work has been devoted almost entirely to his family has prevented him from being known as intimately as many other less interesting and less worthy scientific men. This in spite of the fact that his easy manner and rare conversational gifts ensure the success of any social gathering of which he is a member.

Aside from his existence, it is probable that Doctor Lochhead owes much to his parents, the distinguished and admired William Lochhead and his mother, Lillias Grant, for whom he cherished the warmest affection. He was born on June 21, 1890, at Galt, Ont. After attendance at public school in Guelph and the Guelph Collegiate, he was enrolled at the Montreal High School. In 1911 he graduated from McGill University with honors in chemistry and was awarded an M.Sc. by the same institution in 1912. In the autumn of 1912 he enrolled in the University of Leipzig as a student of Prof. F. Löhnis and, two years later, completed the requirements for the Ph.D. degree. While he was waiting to receive his diploma, war broke out.

The next chapter embraces a romance worthy of the pen of Henry Fielding. In 1913, after graduation from the University of New Brunswick, Helen Louise Van Wart of Fredericton, N.B., went to Germany for advanced musical study at the Leipzig Conservatorium. There she met Lochhead. A mutual attraction developed, and anyone who knows both will not attribute this to loneliness and boredom in a foreign country! On July 30, 1914, Miss Van Wart left for a holiday in England and, not without inconvenience, arrived on August 4. After a few months of study in New York, she returned to Fredericton and taught music for "the duration". Her correspondence with Lochhead has not been made available to this biographer!

After temporary detention in four different internment camps, Lochhead, in November 1914, was assigned to the civilian prisoners' camp at Ruhleben. This was a famous race track where the habitations were designed not for men but for horses. Prisoners were accommodated in haylofts and stalls. Similarly, the food was neither abundant nor palatable.

Thanks to the generosity of British friends, a library of some 4000 volumes was assembled. A biological laboratory was set up in a hayloft. Modest but invaluable equipment for microscopic work, including 10 microscopes, was available, and friends, both British and German, contributed various

<sup>1</sup>Contribution from his colleagues in Science Service, Canada Department of Agriculture, Ottawa.

biological materials. Courses of lectures were arranged, including, for example: *Radioactivity*, by J. Chadwick; *Heredity*, by Michael S. Pease; and *Bacteriology*, by A. G. Lochhead.

During this time Lochhead also acquired a fair knowledge of Italian, which he later taught in the Camp School.

Upon his return to Canada in December 1918, Lochhead was appointed Lecturer in Bacteriology at Macdonald College. On the basis of his work at Leipzig, he was awarded the degree of Doctor of Philosophy by McGill University in 1919. In December of that year, he and Miss Van Wart were married.

Beginning in 1919, Doctor Lochhead spent three years in chemical and bacteriological work, first with Canadian Milk Products in Toronto and then with the Malt Products Company of Canada at Guelph. Following this he was associated with Dr. J. B. Collip at the University of Alberta as lecturer in biochemistry. In 1923 he was named head of the newly created Division of Bacteriology of the Dominion Department of Agriculture, a position he relinquished in 1955. He is now in the happy position of being able to pursue his researches free from the irritations of administrative interruptions.

Before outlining the main features of Doctor Lochhead's researches, it is fitting to record that he and Mrs. Lochhead have two sons. Douglas Grant Lochhead, B.A. (McGill), M.A. (Toronto), B.L.S. (McGill), is Librarian of Dalhousie University, Halifax. Kenneth Campbell Lochhead studied art at the Ottawa Technical School and at Queen's University, after which he spent four years studying at the Pennsylvania Academy of the Fine Arts and attended lectures at the Barnes Foundation, Merion, Pa. He is now Director of Art at Regina College.

Doctor Lochhead is a leading world authority in the field of agricultural microbiology, particularly soil bacteriology. He is author or co-author of over eighty scientific papers. His well deserved reputation is the result of a rare combination of talents. Besides being an acute observer and a skilful technician, he is gifted with sharp critical faculties and originality of thought. Early in his career, he achieved well merited recognition for his researches on the microbiology and fermentation of honey and on the classification, nutrition, and metabolism of osmophilic yeasts. Other contemporary studies included soil bacteriology, the etiology of European foulbrood disease of the honeybee, legume inoculants, water pollution, and the detection and cultural characterization of *Bacillus larvae*, the agent of American foulbrood. These studies, together with investigations on halophilic bacteria causing red discoloration of salted hides, bacteriological examination of meats, and the microbiology of fruits and vegetables, set a pattern for the research work of the Division that, in all essentials, is unchanged today.

Doctor Lochhead's most significant contributions to scientific knowledge have arisen from his studies on soil bacteriology. From the beginning he was keenly interested in the classification of soil-inhabiting bacteria and in methods for studying them. He wrote the section on the genus *Arthrobacter* in the seventh edition of Bergey's "Manual of Determinative Bacteriology", and his

series of papers on "Qualitative Studies of Soil Microorganisms", 13 of which are already published, is known to all who are likely to read this brief review. Included in this series are studies on the microflora of the rhizosphere, the nutritional requirements of soil bacteria, the bacterial equilibrium in soil, and, finally, growth factor production by, and requirements of, soil bacteria.

The study of relationships among the various types of soil bacteria was a natural sequel to their identification. This extremely complex subject was naturally appealing to a man whose work could never be superficial. It was clear that both associative and antagonistic phenomena were involved. Doctor Lochhead has been particularly interested in the former, but he has also contributed much to current knowledge of the production of antibiotics by fungi and actinomycetes and of cross-antagonisms among the latter group of organisms. His interest in associative interactions led to qualitative studies on rhizosphere microflora, the nutritional requirements of the predominant bacterial flora in soil, and, finally, to his most recent contributions on growth factor production by, and requirements of, these bacteria.

Doctor Lochhead's investigations on the rhizosphere indicated that not only do plant roots support larger numbers of organisms but that a selective action is exerted on the organisms in this region, favoring forms with greater physiological activity, such as those attacking glucose as well as liquefying and chromogenic types and Gram-negative rods. These observations have been verified recently through another line of investigation both at the Bacteriology Division and abroad. Further investigations revealed differences in the bacterial flora of the rhizospheres of plant varieties resistant and susceptible to soil-borne diseases and suggested that the selective action of root excretions on the saprophytic soil microflora may be associated with resistance. These results also contributed to the idea of controlling soil-borne diseases by displacing the biological equilibrium in the rhizosphere so as to favor "benign" organisms and inhibit undesirable forms, and led to the work on strawberry root rot, potato scab, and related problems.

His investigations on the nutritional requirements of soil bacteria which resulted in the present classification scheme represent another attempt to characterize the soil microflora itself as well as the microflora of the rhizosphere. The method consists of isolating soil bacteria from representative sections of plates, or from entire plates containing a non-selective (soil-extract) agar medium, and determining their ability to grow on media of increasing complexity, such as those containing simple inorganic salts, amino acids, vitamins, yeast extract, soil extract, or yeast plus soil extract. Aside from providing a new approach to soil microbiology, this led to the significant discovery that organisms requiring amino acids for maximum growth were selectively stimulated in the rhizosphere whereas bacteria with more complex nutritional needs were relatively more abundant in soil at a distance from the root. Studies on excretion of amino acids and other substances by plant roots and by associated bacteria followed naturally and are still being carried on by various members of the staff.

To proceed from studies on amino acid production by soil bacteria to a consideration of the vitamin production by, and requirements of, these organisms was a logical development. There resulted a significant series of papers on the synthesis of vitamin B<sub>12</sub> by soil bacteria, on the production by certain bacteria of the "terregens factor", and, most recently, on the requirements by appreciable numbers of these organisms for these, as well as other, growth-promoting substances and known vitamins. As a result of this work Doctor Lochhead suggested that the importance of microbial antagonisms in soil may have been overemphasized during the past few decades and that the synthetic activities of soil organisms, resulting in the formation and elaboration of vitamins, amino acids, and other nutritionally important substances which are required by various types of soil microorganisms, may be as important a phenomenon as the former. This concept has innumerable practical and theoretical implications. A practical development of these studies was the discovery of bacteria with such specific requirements for vitamins such as B<sub>12</sub> and folic acid that they are used in microbiological assays of these substances. In fact, laboratories in the United States and in England are actually clamoring for more such bacteria and urging that this type of work be continued vigorously.

Doctor Lochhead is a member of many scientific bodies including the Society of American Bacteriologists, Canadian Society of Microbiologists, Society for General Microbiology, Society of Applied Bacteriology, and the Canadian Public Health Association. He was Chairman of the Division of Agricultural and Industrial Bacteriology of the Society of American Bacteriologists in 1953 and, among other committees, is at present also a member of the Canadian Committee on Culture Collections of Micro-organisms and a member of the International Committee on Bacteriological Nomenclature of the International Association of Microbiologists. In recognition of his scientific contributions he has been made a Fellow of the A.A.A.S. and in 1940 became a Fellow of the Royal Society of Canada. He was honored recently by his Canadian colleagues by being elected to the presidency of the Canadian Society of Microbiologists for 1953-1954.

Doctor Lochhead is blessed with a rare combination of scientific and personal qualities. He enjoys the respect and affection of his colleagues all of whom hopefully regard his "extension" as a life sentence.



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## LIFE CYCLES IN *ARTHROBACTER PASCENS* AND *ARTHROBACTER TERREGENS*<sup>1</sup>

C. E. CHAPLIN

### Abstract

Two soil organisms, *Arthrobacter pascens* and *Arthrobacter terregens*, the first producing a growth-promoting substance, the "terregens factor", and the second requiring it, pass through a complex morphological life cycle. Two kinds of aged cells are found of which, on transfer to fresh medium, one forms a cystite which 'germinates' and looses free cells from a ruptured tube, the other follows the usual course of 'normal' cell division. The similarity in form to that of the type species *Arthrobacter globiforme* is quite distinct.

In a study of bacterial growth-promoting substances in soil, Lochhead and Burton (7) recently isolated and described two soil organisms, *Arthrobacter pascens*, capable of producing a previously undetected growth factor, and *Arthrobacter terregens*, which required this factor for growth. For the latter species the "terregens factor" synthesized by *A. pascens* was able to replace the growth-promoting effect of soil extract previously considered essential. These organisms resemble the type species of the genus, *Arthrobacter globiforme*, in showing pleomorphism characteristic of the 'soil diphtheroids'. Descriptions of *Arthrobacter globiforme* (Conn (2, 3) and Taylor and Lochhead (11)), *Corynebacterium helvolum* (Jensen (5)), *Arthrobacter citreus* (Sacks (9)), and *A. oxydans* (Sgueros (10)) have, in common, recognition of an irregular rod, Gram-negative to Gram-variable, becoming a Gram-variable to Gram-positive coccus later in its growth cycle. The formation of cystites mentioned by Jensen also plays a part that is not clear and may be confused by artefact. In preliminary studies *A. pascens* appeared to undergo regular and profound cyclical changes in morphology. This report is a description of the life cycles exhibited by the two species.

### Materials and Methods

The medium was soil extract, prepared according to the description of Lochhead and Burton, to which 0.1% yeast extract was added; it was used either in liquid form, to prepare inocula, or with 1.5% agar for plating. Three

<sup>1</sup>Manuscript received December 17, 1956.

Contribution No. 403 from Bacteriology Division, Science Service, Canada Department of Agriculture, Ottawa.

or four drops of a liquid culture were placed on the surface of a plate and distributed as evenly as possible with a wire spreader. Cultures were incubated at 27° C. Preparations for microscopic examination were made by impressing on to a cover slip the growth on the surface of an agar block cut from a plate of this medium.

Films for cell wall and cytoplasmic staining were fixed in methanol-formalin (90/10 : v/v) and then treated with tannic acid - basic fuchsin, 0.05%, and Azure A, 2%, respectively. They were then mounted in water and photographed immediately. Gram-stained preparations were made in the usual manner.

### Observations

#### *Arthrobacter pascens*

The aged cells used for the inoculum were of two fairly easily distinguishable types: A large majority, perhaps 95%, though no accurate count was attempted, were small and oval with weak staining reaction. They resembled typical micrococci in grouping in a dried preparation from a liquid culture (Fig. 1). The remaining small percentage were larger, more basophilic, spherical cells. The two types are seen in Fig. 2. Within 2 hours of transfer to fresh medium the small cells swelled (Figs. 3, 4) and within 6 hours showed signs of division (Figs. 5, 6, 7). Thereafter their course was an uneventful sequence of cell division with constant decrease in size until the aged appearance was attained in 48 hours (Figs. 8-11).

The larger cells, or cystites (Fig. 12), did not behave in this way. Asymmetrical swelling was the first visible sign that their cycle was more complex (Fig. 13). Usually at 3 hours these cells produced a tube followed shortly by another and often two, so that at 5 hours their appearance was tripodal (Fig. 14). These probably are the structures described by Conn (2). The cystites then divided in such a way that the resulting fragments appeared to be long rods each with a swollen end, these ends being a segment of the original cystite. This division can be seen at various stages in Fig. 15. Shortly thereafter cross walls became visible (Fig. 16) but cell division did not occur by fragmentation of the rod. Instead, the tube ruptured losing small oval cells and leaving an empty tube which could only with difficulty be made visible by tanning and prolonged staining (Fig. 17). It was presumed that the cells so released from the cystite tube underwent no further morphological change in their aging environment.

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#### Photomicrographs (ca. 1740X) of *A. pascens*.

FIG. 1. Gram. 8 days. Typical "micrococcus"-like grouping. FIG. 2. Azure. 12 days. Some "pre-cystite" cells can be seen among the less basophilic majority. FIG. 3. Azure. FIG. 4. Gram. 2 hours. Cells swollen. Cells appear larger and more evenly stained with azure. FIG. 5. Azure. FIG. 6. Tannic acid - basic fuchsin. FIG. 7. Gram. 6 hours. Irregular rods, well formed. FIG. 8. Tannic acid - basic fuchsin. 8 hours. Cross walls formed; cell division advanced. FIG. 9. Tannic acid - basic fuchsin. 18 hours. Small rods. FIG. 10. Gram. 24 hours. Oval rods or cocci. FIG. 11. Tannic acid - basic fuchsin. 48 hours. Coccoid cells.

PLATE I

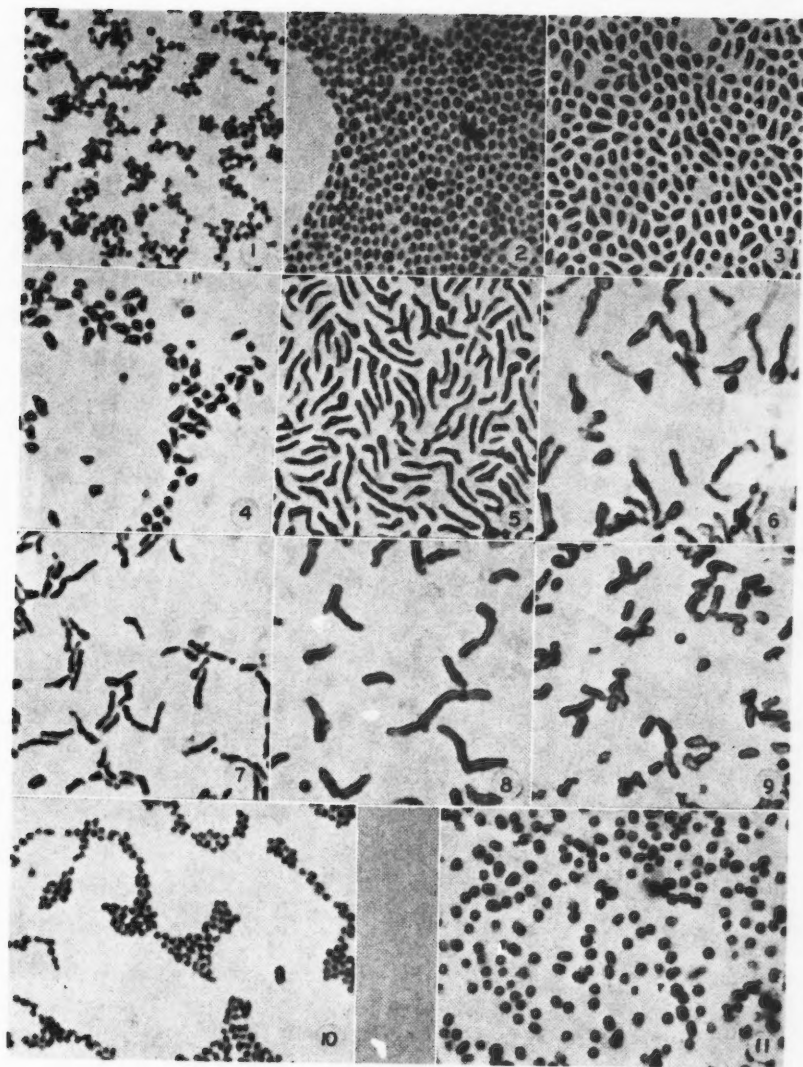


PLATE II



The presence of the cystite tube was deduced from the related behavior of chains of cells as viewed under dark phase microscopy. Each individual cell of a chain would respond in Brownian movement without affecting the position of its neighbors, indicating the cells were not joined directly together, but the movement of the whole chain was such that the relative position of the cells remained unchanged. Following this observation on a culture of *A. pascens*, a more rigorous staining treatment was used and the tubes became visible. A similar treatment of *A. terregens* failed to make the tubes visible. The formation of the complete cells later loosed by the rupturing cystite tube is unusual in bacteria and if confirmed as a constant group characteristic would be a valuable taxonomic feature.

#### *Arthrobacter terregens*

A sequence of events similar to that observed in *A. pascens* was observed in the life cycle of *A. terregens* but the progress from one form to the next was accomplished more slowly and less synchronously. At each stage the cells were smaller than those of *A. pascens*. Fig. 18 shows cells 4 hours after transfer; some swelling had taken place, and 2 hours later (Fig. 19) a characteristic cuneiform cell made its appearance. At 16 hours (Figs. 20-22) there were some cystites and "germinated" cystites and this condition was not much changed 6 hours later. By 40 hours the predominating forms were small spherical to oval cells (Figs. 23, 24) which when dried in a film for Gram-staining assumed the appearance of short irregular rods (Fig. 25). No empty cystite tubes were seen but their presence was assumed.

### Discussion

Comparison of Gram-stained preparations with undried impression films mounted in water illustrates the shrinkage that takes place during the former treatment and, especially in the earlier phases, shows that certain features visible in the chemically fixed preparations will go unnoticed when the cells are distorted by drying in preparation for Gram-staining. In the present instance this is particularly true of the tripodal cystite. The thickness of the cell mass effectively robs the photomicrograph of clarity because it is greater than the depth of focus to which the oil immersion lens is restricted. However, the micrograph of the tannic acid - basic fuchsin preparation at least creates

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Photomicrographs (ca. 1740X) of *A. pascens* (Figs. 12-17) and *A. terregens* (Figs. 18-25).

FIG. 12. Tannic acid - basic fuchsin. 1 hour. Swelling cystite. FIG. 13. Tannic acid - basic fuchsin. 3 hours. "Germinating" cystites. FIG. 14. Tannic acid - basic fuchsin. 5 hours. Tripodal cystites. FIG. 15. Azure. 5 hours. Same stage as in FIG. 14; fragmenting cystites. FIGS. 16 and 17. Tannic acid - basic fuchsin. 24 hours. Cystite tubes with cells inside and after escape of cells. FIG. 18. Tannic acid - basic fuchsin. 4 hours. Cells beginning to swell. FIG. 19. Tannic acid - basic fuchsin. 6 hours. Cuneiform cells at same stage in cycle as cells in FIGS. 3 and 4. FIG. 20. Azure. FIG. 21. Tannic acid - basic fuchsin. 16 hours. Cystites forming and germinated. FIG. 22. Tannic acid - basic fuchsin. 22 hours. Mixture of cell types. FIG. 23. Azure. FIG. 24. Tannic acid - basic fuchsin. FIG. 25. Gram. 40 hours. Cells have assumed aged appearance. In FIG. 25 shrinkage of cells and clumping during drying produced appearance of small irregular rods.

the impression of a structure not confined to the plane of the cover slip. The cytoplasmic stain at the same stage fails to give the impression of depth, and the Gram-stain gives only a slight indication of this structure.

Branching forms mentioned by Jensen have not been seen in the experimental conditions described. The possibility exists that they are a result of the nutritional environment since branching has been shown to occur with another species of *Arthrobacter* in a vitamin-B<sub>12</sub>-deficient medium (1).

Löhnis (8) has defined gonidia as "organs of asexual reproduction formed by the contraction of the plasmatic cell content, which leave the parent cell either by breaking the cell wall, or which become liberated when the cell dissolves", and 30 years later Knaysi (6) remarked "although the existence of gonidia remains a possibility it has never been experimentally proved." Since the definition of gonidia refers only to the method of production and not to any other property of the cell it is felt that the cells formed within the cystite tube may properly be called gonidia.

The life cycles described here show a greater complexity and regularity than was previously assumed in the genus. As the cells described by Conn and Dimmick (4), in their report of the morphological variations of related organisms, were 24 hours of age or older, the description consequently lacks the earlier and more striking changes. However these authors described the cystites and their illustrations show that species differ in the time required to pass through the different stages of the cycle.

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## CHARACTERISTICS OF BACTERIA REQUIRING THE TERREGENS FACTOR<sup>1</sup>

MARGARET O. BURTON

### Abstract

Six organisms belonging to the genus *Arthrobacter* and showing a requirement for the terregens factor (TF) have been studied and compared with the original test organism, *Arthrobacter terregens*. All seven organisms are alike in that they are stimulated not only by the terregens factor but also by certain related substances that contain iron, namely coprogen, ferrichrome, and hemin. TF response is also obtained with aspergillic acid, soil extract, and liver extract. On the basis of their physiological characteristics and nutritional requirements, the organisms were grouped into four types. Growth curves of *A. terregens* and one other organism, based on response to TF, suggest their suitability for use in the microbiological assay of the factor.

Nutritional studies on the indigenous soil microflora have revealed that organisms occur which require for growth a substance known as the terregens factor (3, 6), a heat-stable, water-soluble material not identical with any of the present known B vitamins. This factor, in combination with other known growth-promoting substances, is capable of replacing the aqueous extracts of soil previously found essential to these organisms (8). From culture filtrates of *Arthrobacter pascens*, isolated from soil and found to synthesize TF (6), relatively pure extracts of the factor have been obtained, concentrates of which are active for the test organism (*A. terregens*) at levels as low as 0.001  $\mu$ g. per ml. (3). A recent survey on the occurrence in soils of bacteria requiring the terregens factor revealed that they may attain numbers as high as 500,000 per gram of soil (7). The present study describes seven soil isolates, including *A. terregens* (2, 6), which show a requirement for the terregens factor.

### Isolation of Cultures Requiring the Terregens Factor

Cultures were screened from large numbers of isolates obtained during a series of investigations on the nutrition of soil bacteria.\* For such studies soils were plated on a non-selective soil extract medium and the entire population of a plate or of a suitable sector classified by the nutritional method of Lochhead and Chase (9). For this procedure the isolates were cultured on seven media increasing in complexity from a simple salt-glucose solution to a medium containing yeast and soil extracts (medium YS). Organisms belonging to the S or YS groups, namely those dependent upon soil extract or upon yeast and soil extracts for their nutritive requirements,

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\*The author is indebted to Dr. J. W. Rouatt and Dr. T. M. B. Payne for isolates made available from other research projects.

were further tested to determine those isolates for which the terregens factor could replace the essential ingredient in soil extract. The following three media were employed:

*Yeast extract vitamin B<sub>12</sub> medium (medium YB<sub>12</sub>)*.—K<sub>2</sub>HPO<sub>4</sub>, 1.0 g.; KNO<sub>3</sub>, 0.5 g.; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2 g.; CaCl<sub>2</sub>, 0.1 g.; FeCl<sub>3</sub>·6H<sub>2</sub>O, 0.01 g.; glucose, 1.0 g.; yeast extract (Difco), 1.0 g.; vitamin B<sub>12</sub>, 2.0 µg.; distilled water, 1 liter. The mineral salts are dissolved and the pH of the solution brought to 6.8. The solution is heated to boiling, then cooled before being filtered. The glucose, yeast extract, and B<sub>12</sub> are then added and the pH adjusted to 6.8.

*Yeast extract, vitamin B<sub>12</sub>, terregens factor medium (medium YB<sub>12</sub> TF)*.—Medium YB<sub>12</sub> as above, plus TF to give a concentration of 0.1 µg./ml.

*Yeast extract, soil extract medium (medium YS)*.—The inorganic constituents of medium YB<sub>12</sub> are dissolved and the solution heated to boiling, filtered hot, adjusted to pH 6.8, and the volume brought to 1 liter. To 750 ml. of this solution are added 250 ml. soil extract, 1.0 g. yeast extract, and 0.75 g. glucose.

Medium YB<sub>12</sub>, the negative control medium, was nutritionally complete for the TF-requiring organisms with the exception of the factor itself, which was supplied in the test medium, medium YB<sub>12</sub>TF. Medium YS served as positive control. The organisms for which soil extract was found essential in the original screening were cultured in these three media and incubated at 26° C. for 5 days. Those which showed no growth in medium YB<sub>12</sub>, but maximum growth in both YB<sub>12</sub>TF and YS, were selected as TF-requiring organisms. Six isolates were obtained by this procedure (Nos. 39, 47, 401, 575, 289, 107) for comparison with No. 88 (*Arthrobacter terregens*).

### Growth Response to the Terregens Factor and Related Compounds

In a previous publication the terregens factor was reported to contain no iron but to combine readily with ferrous or ferric iron to form complexes with this metal (3). A number of other substances which either contain iron or combine with it had been shown to be active for *A. terregens*, namely the pigments coprogen (4, 5) and ferrichrome (10), aspergillilic acid, and hemin. In the present investigation, these compounds, as well as liver extract, soil extract ash, and the terregens factor were added respectively to a basal medium of salts, glucose (0.1%), and yeast extract (0.1%). Duplicate tests were carried out in 50 ml. Erlenmeyer flasks containing 10.0 ml. of medium, in shake culture at 26° C. Washed inocula were employed and turbidity readings made after 48 hours' incubation using a Klett-Summerson photo-electric colorimeter at a wave length of 450 mµ. The results are recorded in Table I. Maximum growth by the seven organisms was obtained with TF, coprogen, and ferrichrome at the 0.1 µg./ml. level and, with the exception of No. 39, with aspergillilic acid at 1.0 µg./ml., the concentrations found previously to give optimum growth of *A. terregens* (3). Although both hemin and liver extract showed some stimulation of the seven organisms at the optimum concentration for *A. terregens*, the growth obtained was well below maximum. Soil extract ash did not appear to contain the active principle.



TABLE I  
GROWTH RESPONSE TO THE TERREGENS FACTOR AND RELATED SUBSTANCES

Addenda to basal medium*	TF-requiring organisms						
	88	575	47	107	289	401	39
None	7†	43	37	17	39	5	3
TF (0.1 µg./ml.)	276	217	285	286	280	345	245
Coprogen (0.1 µg./ml.)	293	217	273	267	273	323	288
Ferrichrome (0.1 µg./ml.)	313	211	280	286	280	340	304
Hemin (0.1 µg./ml.)	24	160	54	34	54	33	27
Aspergill acid (1.0 µg./ml.)	272	208	283	286	273	343	166
Liver extract (0.0025 ml./ml.)	68	91	129	89	112	93	111
Soil extract ash (= 25% soil extr.)	8	31	19	9	24	10	16

\*Basal medium: salts, glucose (0.1%), yeast extract (0.1%).

†Average of duplicate tests, turbidity measurements with Klett-Summerson photoelectric colorimeter.

### Vitamin Requirements

Nutritional studies with the TF organisms revealed that the yeast extract of medium YB<sub>12</sub>TF could be replaced by Difco vitamin-free casamino acids (0.1%) supplemented with amino acids *dl*-tryptophan (0.01%) and *l*-cysteine HCl (0.01%) and the following vitamins per 100 ml. of medium; thiamine, 50 µg.; calcium pantothenate, 50 µg.; biotin, 0.1 µg.; folic acid, 10.0 µg.; pyridoxine, 50 µg.; pyridoxal, 50 µg.; pyridoxamine, 10 µg.; riboflavin, 50 µg.; nicotinic acid, 50 µg.; inositol, 5 mg.; choline, 2 mg.; and *p*-aminobenzoic acid, 50 µg. In preliminary qualitative tests in which vitamins were omitted singly or in groups an absolute requirement for thiamine, biotin, and TF was demonstrated by all seven organisms. However, calcium pantothenate was important in the nutrition of Nos. 88 and 575, being essential for the former and noticeably stimulatory to the latter. Culture No. 39 differed from the others in requiring *p*-aminobenzoic acid.

Quantitative tests with the seven organisms were then carried out by employing the casamino acid medium described above with only the essential vitamins added. Turbidity measurements of duplicate flask tests were made after 72 hours' incubation as recorded in Table II. Calcium pantothenate, although not essential to No. 575, was found to be required for optimum growth. With organism No. 39, a response was obtained when *p*-aminobenzoic acid was replaced by folic acid, a substance which contains the former vitamin within its structure (11).

### Description of Organisms

The differential media employed in the morphological and physiological tests were prepared by standard procedures and supplemented with the terregens factor either by the substitution of soil extract for water or by the addition of 0.1% of a culture filtrate of *A. paszens*. In certain media 0.1% yeast extract was also included to provide additional vitamins. Stock cultures

Vitamin additions to basal medium*	TF-requiring organisms						
	88	575	47	107	289	401	39
None	4†	16	11	9	10	18	2
B <sub>1</sub> , biotin, TF	10	105	274	295	299	268	7
B <sub>1</sub> , biotin, TF, Ca pantothenate	310	221	280	277	266	270	5
B <sub>1</sub> , biotin, TF, p-aminobenzoic acid	9	80	284	289	278	277	238
B <sub>1</sub> , biotin, TF, folic acid	10	112	283	284	293	269	110
13 B-vitamins, TF	320	227	282	256	265	280	236

†Average of duplicate tests, turbidity measurements with Klett-Summerson photoelectric colorimeter.

On the basis of their morphological and physiological characteristics, the seven organisms were considered to be representative of the genus *Arthrobacter* (1). They demonstrated the characteristic pleomorphism of the soil corynebacteria, changing from rod forms of the 'diphtheroid' type in young culture to coccoid forms in older cultures. All the organisms were Gram-variable, strongly aerobic, and non-motile, producing little or no acid in carbohydrate media and forming no indol from tryptophan. However, the seven organisms could be separated into four types depending upon their general physiology and nutritional requirements as summarized in Table III.

	TF-requiring organisms						
	88	575	47	107	289	401	39
Pigmentation (agar)	Yellowish brown	Pale yellow	Creamy pale yellow	Creamy yellow	Creamy pale yellow	Colorless or very pale yellow	Colorless
Gelatin liquefaction	—	—	+	+	+	+	+
Milk	No change	No change	Casein dig.	Casein dig.	Casein dig.	Casein dig.	Casein dig.
Glucose	No change	Sl. acid	Acid	Acid	Acid	No change	Acid
Sucrose	No change	No change	Acid	Acid	Acid	Acid	Acid
Lactose	No change	No change	Alk.	Alk.	Alk.	Alk.	Sl. acid
Starch hydrolysis	—	+	—	—	—	+	—
Nitrate reduction	+	+	—	—	—	+	—
Growth at 37° C.	—	—	+	+	+	+	—
Vitamin requirements	B <sub>1</sub> Biotin Ca pant.	B <sub>1</sub> Biotin Ca pant.	B <sub>1</sub> Biotin	B <sub>1</sub> Biotin	B <sub>1</sub> Biotin	B <sub>1</sub> Biotin	B <sub>1</sub> Biotin <i>p</i> -Aminobenzoic acid

Number 575 is closely related to No. 88 (*A. terreus*), differing only in its ability to hydrolyze starch and by showing a marked stimulation effect with calcium pantothenate rather than an absolute requirement for that vitamin. A second group consisting of Nos. 47, 107, and 289 showed the yellow-brown pigmentation of Nos. 88 and 575, differing from these organisms, however, in vitamin requirements and certain physiological characteristics. These three cultures having similar characteristics are probably the same species. Number 401 demonstrated greater biochemical activity than the other organisms tested, being able to digest casein, hydrolyze starch, liquefy gelatin, reduce nitrates, and show growth at 37° C. Number 39, a non-pigmented organism, in addition to differing in some physiological characteristics from the others studied, showed a specific requirement for *p*-aminobenzoic acid.

#### Cultures 88 and 401 as Assay Organisms for TF

To compare their suitability as assay organisms for the terreagens factor, tests were made with No. 88 (*A. terreus*), the original TF-requiring organism, and No. 401, which was found to be the most physiologically active of the seven bacteria responding to the factor. A medium of the following composition was used: basal salts as in medium YB<sub>12</sub>; glucose, 1.0 g.; yeast

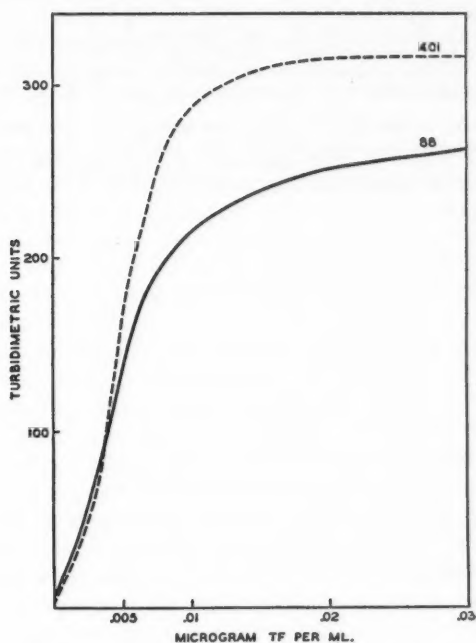


FIG. 1. Growth responses of cultures Nos. 88 and 401 in relation to terreagens factor concentration.

extract, 1.0 g., and distilled water, 1 liter. The assay procedure was that outlined in a previous publication (3), employing, however, the following levels of TF per ml.: 0, 0.001, 0.0025, 0.005, 0.0075, 0.01, 0.02, and 0.05  $\mu$ g. Turbidity readings were made after 48 hours' incubation. Typical growth curves are shown in Fig. 1 where it will be seen that with both organisms maximum density is obtained with approximately 0.02  $\mu$ g. TF per ml. of medium. However, organism No. 401 showed a higher turbidity than No. 88 within the 48 hour growth period and a suitably low response in the absence of the factor, suggesting its value for use in assaying the terregens factor.

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## NODULATION RESPONSES OF TWO NEAR ISOGENIC LINES OF THE SOYBEAN<sup>1</sup>

FRANCIS E. CLARK

### Abstract

Nodulation responses and certain other characteristics of a mutant soybean line highly recalcitrant to nodulation were compared with those of a nodulating sister line. Roots of the two lines were found to harbor equal numbers of rhizobia. Stem graftings to provide top growths of one line on roots of the other failed to alter the distinctive nodulation responses of rootstocks. Ascorbic acid contents in the two lines were identical, both in the tops and in the roots, although contents in tops greatly exceeded those found in roots. Chromatographic studies on the amino acids in seed hydrolyzates and in alcoholic extracts of seedlings showed no differences between the two lines either in kind or quantities of amino acids. In a survey of stock rhizobia for cultures effective on the nonnodulating line, bacteria were discovered which formed nodules on such soybeans growing in sand and nutrient solution. Isolates from these nodules again yielded effective nodulation on plants in sand culture, but gave no nodulation whatsoever on plants growing in soil. This negative response was confirmed in three different soils. Admixtures of soil and of miscellaneous materials with sand were employed to alter nodulation responses from those shown in sand cultures.

### Introduction

Rhizobia which associate themselves with roots of leguminous plants and effect symbiotic nitrogen fixation have been studied more extensively than any other group of soil bacteria. That the legume plant itself plays an active part in determining the occurrence of symbiosis is well established. Much of the information about characteristics of host plants that can be linked to their nodulation responses concerns nutritional or environmental factors that influence nitrogen fixation of plants normally capable of forming nodules. For example, either lack of an essential micronutrient or excess of nitrogen in the substrate is known to inhibit nodulation. Finding the specific factors in plants normally incapable of nodulation that deter them from fixing nitrogen might well prove to be an important step in extending symbiotic fixation to additional species of plants.

The role of genetic factors in the plant in determining the success or failure of nodulation responses has been shown by Nutman (9, 10) and various others. Recently, there has been discovered and propagated a mutant soybean strain which does not form nodules, nor fix atmospheric nitrogen, when grown in soil containing soybean rhizobia (8, 12). The hereditary factor associated with the failure to nodulate behaves genetically as a single recessive. Existence of this nonnodulating soybean and its nodulating sister strain has provided a specific opportunity for investigating characteristics of two closely related soybeans which differ in their ability to fix atmospheric nitrogen in symbiosis with rhizobia.

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### Materials and Methods

The plant material employed has consisted of seeds, seedlings, and plants of two near isogenic lines of soybeans. Both trace back to the same BC<sub>1</sub>S<sub>2</sub> plant from a Lincoln × (Lincoln × Richland) backcross made in 1943 (12). For convenient and easy identification, the nodulating sister strain is designated as *NN*, and the nonnodulating strain as *nn*. These lines have been crossed to give the two used in this study. For practical purposes the two lines are now essentially identical in genetic make-up except for the single gene under study and possibly a few additional genes closely linked with it.

All seed supplies were obtained from Dr. C. R. Weber, Iowa Agricultural Experiment Station, Ames, Iowa.

Microbiological plating techniques were employed to determine occurrence and number of rhizobia and the total number of microorganisms associated with the root systems of the nodulating and nonnodulating soybeans. Rhizobia were enumerated on yeast-extract mannitol agar and total bacterial numbers were determined on soil extract agar.

Some 80 strains of rhizobia were tested for their ability to cause nodulation in the *nn* and *NN* soybeans. All rhizobial strains were made available by Dr. L. W. Erdman of this Laboratory, and strain identification numbers, when used, are his designations. Greenhouse facilities were employed for conducting nodulation tests. For the majority of tests, soybeans were grown in sand and nutrient solution contained in a glass jar assembly described by Erdman (4). The modified Crone's nutrient solution was a complete nutrient solution except for nitrogen. In a few instances nitrogen was also added. Each inoculation treatment was replicated at least three times. Not all inoculants represented single pure cultures. In the initial survey for effective strains, composite inocula each containing five strains of rhizobia were used.

Nodulation responses were also observed in four different soils, namely, Webster loam, Brookston silt loam, Norfolk sandy loam, and Cecil sandy loam. Soil or sand substrates variously amended with other materials were also employed for soybean cultures in the greenhouse. Amendments included Wyoming bentonite (a montmorillonitic clay), alfalfa meal, and finely ground peat.

Ascorbic acid was determined as follows: 0.5 g. of plant material was triturated in 10 ml. of 5% metaphosphoric acid, and this in turn was shaken with one drop of bromine water and aerated to remove the excess bromine. Solid thiourea was added to make 1%. One milliliter of 2% solution of 2,4-dinitrophenylhydrazine in 9 *N* sulphuric acid was added to 4 ml. of the unknown. The mixture was held at 37° C. for 3 hours, then transferred to an ice bath and given 5 ml. of 85% sulphuric acid drop by drop in 1 minute. Thirty minutes after removal from the bath, light transmission was determined at 5400 Å. Values for ascorbic acid were then read from a standard curve.

For chromatographic detection of amino acids, Whatman No. 7 filter paper, 80% phenol, and a 50:40:10 combination of butanol, acetic acid, and water were found satisfactory. In general, operational procedures for qualitative



paper chromatography were according to Block *et al.* (1, 2), and for quantitative chromatography, according to Fisher *et al.* (5, 6). The method of preparing plant material for free amino acid analysis consisted of grinding or homogenizing the roots or seeds with sufficient absolute ethanol so that the final concentration of the alcohol was 80% by volume. The ethanol extracts were cleared by centrifugation and three volumes of chloroform were added to each volume of cleared extract. After thorough shaking, the upper or aqueous layer was removed and concentrated to the desired volume by evaporation over a steam bath. The preparation was then placed on an Amberlite IR-120 resin column in the H cycle and eluted with 2 *N* ammonium hydroxide. Evaporation to dryness removed the ammonia. Finally, the amino acids were taken up in 2 ml. of 0.1 *N* hydrochloric acid.

For analysis of amino acids in the seed protein, a sample containing 3.5 mg. of protein was hydrolyzed under reflux with 30 ml. of 6 *N* hydrochloric acid for 20 hours. The excess hydrochloric acid was removed by evaporation to dryness *in vacuo* at 35° C. over a steam bath. The residue was then taken up in 2 ml. of water for transfer to the cation exchange resin described above.

## Results

### *Bacterial Compatibility Studies*

Rhizobia were recovered with equal facility and in equivalent numbers from roots of *NN* and the *nn* soybeans, regardless of whether the plants were cultured in the field or in the greenhouse. Employment of aseptic cultural conditions also failed to reveal any differences in the compatibility of soybean rhizobia and roots. Following treatment of seeds with alcohol and mercuric chloride to obtain bacteria-free seed, seedlings of the two lines were grown in Crone's nutrient solution inoculated with rhizobia. There was comparable development of the inoculated bacteria.

For plants growing either in the field or in the greenhouse, total bacteria on the roots of the nodulating soybean were more numerous per gram of root sample than on the nonnodulating roots. The difference in the field was of the order of 20%. Attempts to demonstrate any bacteriostatic activity in the expressed, unheated root juices of either *NN* or *nn* plants were unsuccessful.

It was believed that the *NN* soybean provided the rhizosphere bacteria with a more abundant or a more favorable nutrient supply than did the *nn* plant. The *NN* soybeans as well as their seeds contained more nitrogen. Kjeldahl nitrogen determinations and the use of a conversion factor of 5.71, which according to Jones (7) expresses satisfactorily the relationship between total nitrogen and protein in the soybean, showed that *NN* seeds contained 37% protein, and the *nn*, 31%. The lack of symbiotic fixation in the *nn* plants provides the most obvious explanation for their lower seed nitrogen and protein content. Crude carbohydrate, fat, and ash contents were all 1 or 2% higher in the *nn* than in the *NN* seeds. These differences are understandable in view of the differing protein contents.

### *Plant Grafts*

Plant grafts were made in order to determine whether any substances formed in the tops of either the *nn* or *NN* plants would increase or decrease the susceptibility of roots to rhizobial infection.

Approach-type grafts were made on plants 3 to 4 weeks old by slicing off longitudinally and below the first trifoliate leaf about one-half the stem for a distance of 2 or 3 cm., and then taping together two such stems in order to provide close contact between their cut surfaces. After a week to 10 days, stems were cut above or below the union in order to secure desired root and top combinations. Grafts were made between *NN* and *nn* plants growing in the same container of soil as well as between others growing in separate containers. Observations for nodulation were made 6, 8, or 10 weeks after the initial grafting operation.

Nodulation was absent on *nn* roots grown in combination with *NN* tops. For *NN* roots supporting *nn* tops, there were no differences in size, number, or appearance of nodules compared to those obtained on *NN* control plants. In other grafts in which both *NN* and *nn* roots were retained below, and both tops above the stem union, nodulation responses of *NN* and *nn* roots also agreed with those of the respective controls.

These results are in agreement with those obtained by Nutman (10) working with red clover. He found no transfer across the graft union of any factor influencing the nodulation responses of susceptible and resistant rootstocks. Dissimilar results were obtained by Bonnier *et al.* (3). Nodulation of the roots of soybeans by rhizobia normally ineffective on this legume was obtained by grafting to soybeans other leguminous species on which the rhizobia were normally effective. If their results are accepted at face value, then one must conclude that entirely dissimilar scions can influence nodulation responses of roots, whereas cross-grafting of two very closely related varietal lines exerts no such influence.

### *Ascorbic Acid Determinations*

Tonzig and Bracci (11) have suggested that ascorbic acid is the factor regulating the morphological responses between leguminous plants and rhizobia. They found that both legume roots and tops normally harbor nitrogen-fixing bacteria, but that only the roots, which are exceedingly poor in ascorbic acid, permit the formation of nodules. Failure of rhizobia to cause morphological reactions in the aboveground parts of the plants was believed to be due to the higher ascorbic acid contents of these parts. Roots experimentally supplied with extra ascorbic acid failed to develop nodules. Their work suggested the desirability of comparing the ascorbic acid contents of the *NN* and the *nn* soybeans.

Ascorbic acid determinations were made on 4-day-old seedlings and on plants 2 and 6 weeks old. Table I summarizes the data obtained.

At 4 days, the values for cotyledons and roots were approximately the same. The slightly higher values for the *NN* seedlings may be explained by their



TABLE I  
ASCORBIC ACID CONTENTS OF *NN* AND *nn* SOYBEANS

Age of plants	Plant sample	Ascorbic acid, p.p.m.	
		Variety <i>NN</i>	Variety <i>nn</i>
4 days*	Cotyledons	180	180
	Roots	105	125
2 weeks*	Leaves	515	700
	Roots	68	86
6 weeks*	Leaves	1010	1105
	Roots	255	175
6 weeks†	Leaves	690	620
	Roots	355	240

\*Plants grown in sand fertilized with complete nutrient solution including nitrogen.

†Nitrogen omitted from nutrient solution. *NN* as well as *nn* plants were grown non-nodulated.

higher seed nitrogen contents causing in turn a more vigorous seedling development. For plant samples taken after 2 and 6 weeks of growth, ascorbic acid contents for the *NN* line did not differ significantly from those in the *nn* soybeans. For these samples, ascorbic acid contents in the leaves markedly exceeded those in the roots. This difference within plants was less marked for uninoculated, nitrogen-starved plants 6 weeks old than for well-nourished plants of the same age and growing in a fertile soil.

#### Amino Acid Comparisons

Acid hydrolyzates prepared from either the *NN* or *nn* seeds were found to contain all but one of the amino acids previously reported in the soybean protein (7). The presence of methionine was not established with certainty. Methionine sulphone was found in the alcoholic seed extracts after oxidation with hydrogen peroxide. This suggests that the methionine was lost during acid hydrolysis.

The free amino acids present in soybean seeds, in cotyledons 5 days after germination, and in the roots at 5, 14, and 28 days are shown in Table II.

In neither seeds nor seedlings was there discovered any qualitative difference in the free amino acid composition of the *NN* and *nn* strains. The quantities of the individual amino acids present in the two lines, when estimated on the basis of equal quantities of amino acid nitrogen, were found practically identical. For 10 amino acids for which quantitative estimates were made, the values in grams per 100 g. of oven-dry seed for the *NN* and *nn* seeds respectively were as follows:  $\alpha$ -alanine, 0.8 and 0.7;  $\beta$ -alanine, 0.6 and 0.5; arginine, 3.4 and 2.9; aspartic acid, 1.3 and 1.2; glutamic acid, 4.9 and 4.5; histidine, 1.2 and 1.0; serine, 2.0 and 1.7; threonine, 2.0 and 1.6; tyrosine, 2.1 and 1.6; and valine, 2.5 and 2.0. Leucine, isoleucine, and phenylalanine as a group were determined as 7.9 and 7.0. Cystine, glycine, lysine, proline, and tryptophan were not sufficiently resolved to permit quantitative estimation.

TABLE II

AMINO ACIDS FOUND IN ETHANOL EXTRACTS OF SEEDS, COTYLEDONS,  
AND ROOTS OF *NN* AND *nn* SOYBEANS

Amino acid	Seeds	Cotyledons	Roots		
		5 days	5 days	14 days	28 days
$\alpha$ -Alanine	+	+	+	+	+
$\beta$ -Alanine	+	+	+	+	+
$\alpha$ -Aminobutyric		+	+	+	
$\alpha$ -Aminobutyric		+	+	+	+
Arginine	+	+	+		
Asparagine	+	+	+	+	+
Aspartic acid	+	+	+	+	+
Cystine*	+	+	+		
Glutamic acid	+	+	+	+	+
Glutamine	+	+	+	+	+
Glycine	+	+	+	+	+
Histidine	+	+			
Leucine	+	+	+	+	+
Isoleucine	+	+	+	+	+
Lysine	+	+			
Methionine*	+				
Phenylalanine	+	+	+	+	
Proline	+	+	+	+	
Serine	+	+	+	+	+
Threonine	+	+	+	+	+
Tryptophan	+	+	+	+	
Tyrosine	+	+	+	+	
Valine	+	+	+	+	+

\*Presence of cystine and methionine inferred following detection of cysteic acid and methionine sulphone after treatment with hydrogen peroxide.

The data obtained were sufficient to suggest that the two soybean lines do not differ materially in their protein composition. There were changes in the amino acid composition at differing stages of growth. Some free amino acids were detected in the roots at 5 days that were not found in the seeds themselves. By 28 days, a number of amino acids present at 5 or 14 days could no longer be detected. Whatever changes that occurred in the *NN* soybean were also observed in the *nn* variety; there were no clues to any metabolic differences that could be linked to nodulation failure of the latter variety.

#### Screening Studies with Soybean Rhizobia

The apparent compatibility of rhizobia with roots of the *nn* soybean as well as the failure to detect any ascorbic or amino acid differences between the two lines pointed up the desirability of screening available rhizobia for ability to nodulate the *nn* soybean.

The initial screening experiment involved 74 cultures pooled into 15 inoculation mixtures. Fourteen mixtures each contained five cultures, and the remaining mixture, four cultures. Three soybean plants were grown per container, and there were three replications for each inoculation mixture. Root examinations were made at time of blooming on 136 plants.

Sixty-two of these plants, or 45.6%, were found to be effectively nodulated. Both the number of plants showing nodulation and the irregularity of the nodulation responses were unexpected. It had been surmised that only very few, if any, of the inoculation mixtures would produce nodulation. Actually, 14 of the inoculants proved effective on at least some plants. It was also expected that nodulation, when present, would involve all plants within a single container. Almost without exception this was not the case. With 13 of the inoculants, the number of plants showing nodule formation ranged from one to eight out of a total of nine. One of the two remaining inoculant mixtures nodulated none of the 10 plants on which it was tested; the other nodulated all nine plants to which it was applied.

These results indicated that almost any inoculation mixture was able to nodulate *nn* soybeans. This, however, was not in agreement with our earlier experience with a large number of *nn* plants that were grown in soil for the biochemical and grafting studies, nor with observations reported by agronomists for *nn* soybeans in the field.

In order to confirm this limited but nevertheless apparently successful nodulation of *nn* soybeans, a second experiment was initiated in which plants were cultured in sand-nutrient solution substrate and in three soils, namely, Brookston silt loam, Norfolk sandy loam, and Cecil sandy loam. Seventy-nine strains of rhizobia, the 74 previously used and five additional, were pooled to provide four inoculation mixtures containing 20, 20, 20, and 19 individual strains.

No nodulation whatsoever was observed in the Brookston, Cecil, and Norfolk soils. All companion *NN* plants grown at the same time were profusely nodulated.

In the sand-nutrient solution cultures, 11.3% of all *nn* plants grown were nodulated. Again, positive nodulation was irregular, usually it occurred in only one of three replicate containers and on only one plant. No explanation can be given for the fact that a lower percentage of *nn* plants were nodulated in this than in the preceding experiment. The sand substrates provided are believed to have been identical. One experimental difference was the mixing together of 20 rather than 5 individual rhizobial cultures in the inoculants. Possibly more important was an environmental difference in day length and in temperature. In the first screening, the soybeans were cultured during the early summer, and in the second, during the late fall.

A third experiment was designed to test whether rhizobia obtained from effective root nodules on *nn* soybeans could be used to achieve a more uniform nodulation of all plants within any given inoculation treatment. Eight single strain inoculants, representing rhizobia isolated from nodules on *nn* soybeans

in the two preceding experiments, and two crushed-nodule suspensions were used to inoculate a total of 78 *nn* plants. Of the single strain inoculants, four failed to nodulate any plants; the remaining four caused formation of one or few effective nodules on from 50 to 100% of the plants to which they were applied. One of the crushed-nodule suspensions produced nodules on 12 of 15 plants, and the other on 6 of 15 plants. Nodules, when present, were the large or effective type and showed deep red internal pigmentation.

That these nodules did effect symbiotic nitrogen fixation was shown by Kjeldahl nitrogen determinations that were made on plant material harvested from the experiment just described. The vegetative growths of *nn* soybeans grown (a) uninoculated and nonnodulated, (b) inoculated but showing no nodules, and (c) and (d) inoculated and nodulated were found to contain 1.64, 1.66, 2.05, and 2.99% nitrogen, respectively. The total plant nitrogen in the nonnodulated plants was approximately equal to the seed nitrogen, whereas in the nodulated plants the nitrogen content was fully twice the seed nitrogen.

These data, showing effective nitrogen fixation with rhizobia recovered from root nodules on the *nn* soybean, were sufficiently encouraging that it appeared desirable to test a number of single strain isolates, all from effective nodules on *nn* soybeans, on both *nn* and *NN* plants cultured in sand and in soil.

Each of 18 rhizobial inoculants obtained from effective nodules was applied to seeds of the *nn* soybean. These seeds were planted to provide final stands of four plants per container in sand - nutrient solution substrate. There were five replications. Ten of the rhizobial strains were used to treat additional *nn* seeds for planting in Cecil sandy loam, Norfolk sandy loam, and Brookston silt loam. Five replications were again used. All inoculation treatments made on *nn* soybeans were also made on *NN* soybeans, the only difference being that for the *NN* cultures three and not five replications were employed.

Altogether, 600 *nn* soybeans were cultured in the three soils. Of this total, only one plant was found to be nodulated when the experiment was discontinued at the time plants were in the early pod stage. This exceptional plant had numerous nodules, and there remains the possibility that it grew from an *NN* seed inadvertently present in *nn* seed. Bacteria were isolated from root nodules on this plant for further testing on the nonnodulating soybean. Such tests have not yet been conducted.

Of the 350 *nn* plants grown in sand, 161 were nonnodulated, and 189, or 54%, were effectively nodulated. All plants of the *NN* soybean were nodulated, regardless of whether grown in sand or in soil. Some of the *NN* plants showed parasitic rather than effective nodulation.

A summary of the nodulation responses of the *nn* plants grown in sand to 18 different rhizobial strains is given in Table III.

#### *Nodulation Responses in Sand-Soil Mixtures and in Sand Substrates Variouslly Amended*

The data of Table III again emphasized that consistent nodulation of the *nn* soybean was difficult to obtain. A point of even greater interest in the

TABLE III

SUMMARY OF NODULATION RESPONSES OF *nn* SOYBEANS TO 18 SINGLE CULTURE INOCULANTS

Rhizobium strain No.	Plants nodulated, of total number of plants	Rhizobium strain No.	Plants nodulated, of total number of plants
311b-83a	20 of 20	311b-85a	10 of 20
-84a	20 of 20	-81a	8 of 15
-83	18 of 19	-81	8 of 20
-86	18 of 20	-81b	7 of 18
-86a	18 of 20	-79	5 of 19
-82b	17 of 19	-84	4 of 20
-80	13 of 20	-82	0 of 20
-86b	12 of 20	-82a	0 of 20
-85	11 of 20	-74	0 of 20

greenhouse work generally was the repeated failure to obtain nodulation of the *nn* variety in soil in contrast with the success that could be obtained in sand cultures. This difference in response led to the establishment of soybean cultures in which various other materials were admixed with sand.

In an effort to determine whether or not the difference in nodulation response between sand and soil was gradational, Brookston silt loam was mixed with sand to provide the following percentages of soil in sand: 50, 25, and 12.5. Triplicate containers were also established for soil alone and for sand alone. Additionally, sand cultures were established in which the sand was amended with 2% alfalfa meal, 2% finely ground peat of the quality commonly used as a carrier for commercial legume inoculants, or 10% Wyoming bentonite, a montmorillonitic clay.

All seeds were treated with hydrogen peroxide for 30 minutes, thoroughly washed, and then inoculated with culture 311b-84a, a presumably effective strain of *Rhizobium* (see Table III). Three soybean plants were grown per container. All substrates were kept moistened with the nitrogen-deficient Crone's solution. After 7 weeks, plant roots were washed free of the substrates and the individual root systems were examined for root nodules. The nodulation responses observed are summarized in Table IV.

The differing nodulation response between *nn* soybeans grown in sand and in soil was again apparent, even though in the current test Crone's nutrient solution was used to wet the soil. In previous work Crone's solution had been used to maintain substrate moisture in sand cultures, but not in soil.

The limited testing done with soil-sand mixtures indicates that soil mixed with sand in proportions as low as one part in four or one part in eight is less favorable to nodulation than is straight sand. However, the fact that one root nodule was obtained on one *nn* plant growing in a mixture of half sand and half soil does suggest that continued attention both to substrate conditions and to inoculant selection should in the near future permit the successful nodulation of *nn* soybeans in soil.

No reason has yet become apparent why soil is less conducive to nodulation than is the sand. The fact that alfalfa meal, rich in nitrogen, when added to

TABLE IV

NODULATION OF *nn* SOYBEANS IN DIFFERING SUBSTRATES

Substrate	Plants grown	Plants bearing nodules	Total number nodules	Remarks
1. Sand	12	11	13	Typically one effective nodule per plant
2. Sand: soil as 7 : 1	12	8	8	Typically one effective nodule per plant, if nodulated
3. Sand: soil as 3 : 1	12	3	3	" " " "
4. Sand: soil as 1 : 1	12	1	1	" " " "
5. Soil	12	0	0	—
6. Sand plus 2% peat	12	12	57	Several large effective nodules per plant
7. Sand plus 2% alfalfa meal	12	11	43	Many of the nodules quite small
8. Sand plus 10% Wyoming bentonite	12	0	0	—

sand did not entirely prevent nodule formation, whereas bentonite, lacking in nitrogen, did prevent nodule development, makes it difficult to believe that the small amounts of soil nitrogen that became available in the soil-sand mixtures are responsible for inhibition of nodulation.

Quite surprisingly, the nodulation response obtained in the sand-peat mixture exceeded that obtained in sand alone. Although study of the *nn* soybean has yielded no clue to account for its extreme recalcitrance to nodulation, this study has revealed that the mutant soybean does provide excellent plant material for studying borderline occurrence of nodulation. Further attention to this mutant should prove rewarding to workers interested in the nodulation responses of plants.

#### Acknowledgments

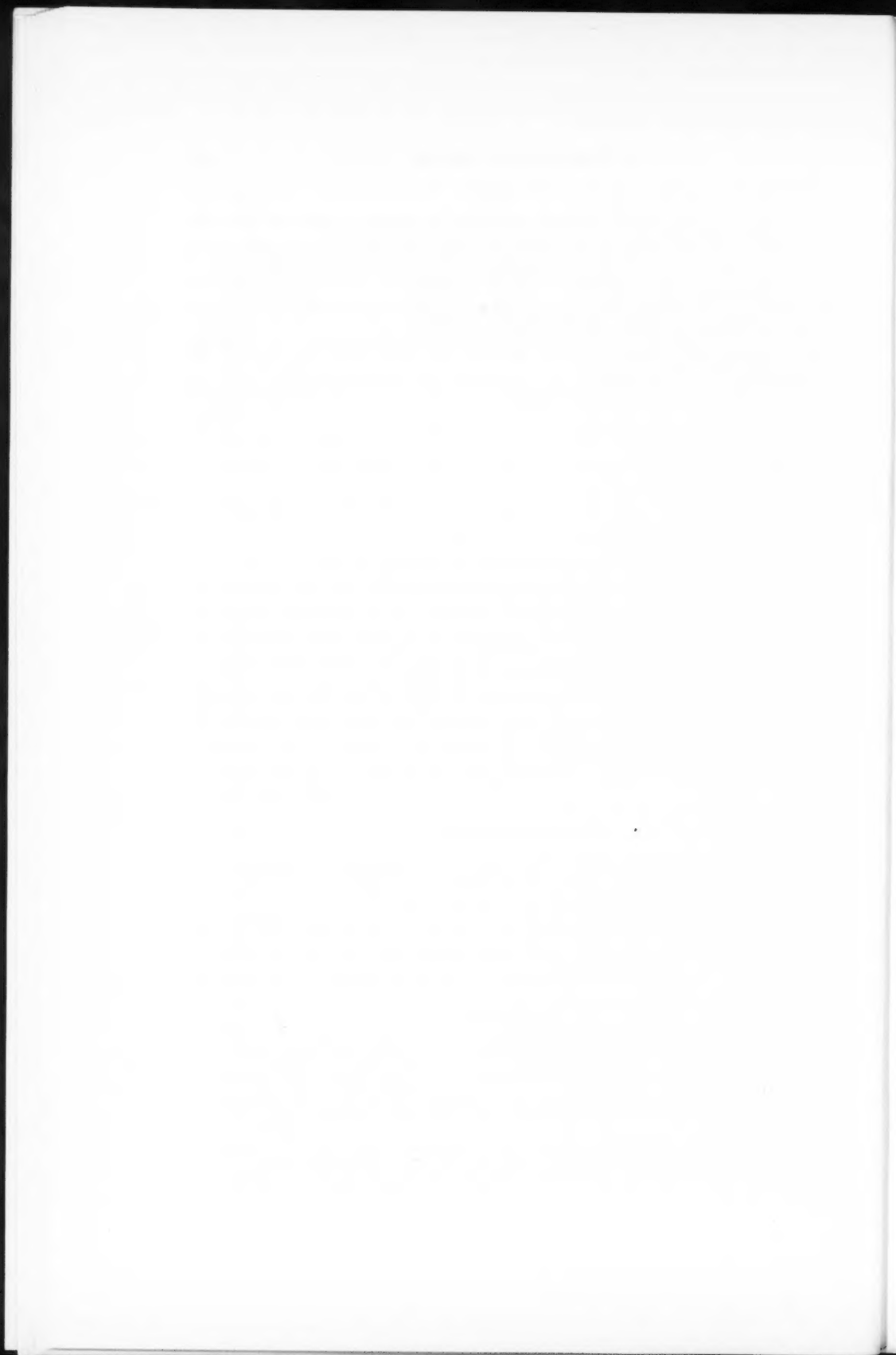
The writer acknowledges deep appreciation to D. C. Nearpass for assistance with the ascorbic acid determinations; to Arnis Kuksis, for the amino acid chromatography; to Doris G. Rankin, for Kjeldahl nitrogen determinations; to Ura Mae Means, for assistance with the bacterial and plant cultures; and to Lewis W. Erdman, for making available an extensive collection of bacterial cultures and in seeking to obtain new and effective isolates.

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## BACTERIA IN CLOVER ROOT TISSUE<sup>1</sup>

M. N. PHILIPSON AND I. D. BLAIR

### Abstract

Detection of bacteria other than *Rhizobium* in nodule cultures led to studies on bacteria existing within normal clover roots. Those isolated from clover root tissue appeared to fall into five groups on the basis of morphology and biochemical characteristics. The identity of three of the groups was tentatively established respectively as *Aerobacter cloacae* Jordan, Bergey *et al.*, *Bacillus megatherium* De Bary, and *Flavobacterium rhenanus* (Migula) Bergey *et al.*

### Introduction

To Perotti (8) in 1926 may be accredited the finding that a non-pathogenic bacterial flora can be defined within root tissue, while Hennig and Villforth (5) later confirmed this when they found bacteria in leaves, stems, and roots of 28 different healthy plants examined by them. Similar results have been obtained separately by Marcus (7) and Sanford (9), *Agrobacterium radiobacter* being found frequently by the latter in plant tissues. Bacteria in stem tissues of potatoes have been described by Tervet and Hollis (10) and by Hollis (6), *Bacillus megatherium* being of common occurrence. A fact revealed by the work of Thomas and Graham (11) was the apparently passive existence in some healthy bean tissues of bacterial types normally pathogenic. All these studies certainly stimulate enquiry concerning environmental or physiological conditions within plants which may influence changes by bacteria from states of commensalism or passivity to pathogenicity. Meanwhile, however, this report is concerned only with evidence of the mixed bacterial flora of clover roots, this situation having been examined as a consequence of difficulties occasionally encountered in obtaining pure *Rhizobium* cultures from surface disinfected root tissues.

### Experimental

Host plant material was used of both red (*Trifolium pratense*) and subterranean (*Trifolium subterraneum*) clover. As the isolation of bacteria from within root tissue might be ascribed to faulty technique, every possible precaution was taken to exclude the ingress of surface contaminants. Root portions, after washing, were surface scraped by scalpel, disinfected in acidulated mercuric chloride, 1 : 1000, and washed in changes of sterile water. Final washing and storage were performed in small, plugged, specimen tubes. Tissue pieces were then transferred aseptically to culture dishes where any remaining epidermal portions were removed. Inner tissue portions were then placed in other sterile dishes and dissected further before transference to stoppered tubes of enriched nutrient broth, as described by Hollis (5). After incubation at 22° C. for 4 days, dilution streaks from tubes showing turbidity

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were made on both yeast extract mannitol agar and to a buffered potato dextrose agar (Hollis (5)). Many bacterial colonies were isolated and eventually subcultured. Some of them were difficult to maintain on agar and appeared to be discriminating in nutritional requirements. Five types were chosen for study because of their vigor and comparative abundance, and the characteristics of these are described hereunder.

In bacteriological studies (below) cultures were incubated at 22° C. Carbohydrate, nitrogenous, and other media were prepared after the methods of Dowson (3). The carbohydrates contained 1% of the carbon compound, with bromocresol purple as indicator, and were at pH 7.0. Hydrogen sulphide tests were performed on nutrient agar containing 0.5% basic lead acetate.

## Results

### CHARACTERIZATION OF ISOLATES

#### *Isolate R.3*

From inner taproot tissue of *Trifolium pratense*. Motile rods with rounded ends 0.3–0.8 × 0.8–1.3  $\mu$ ; single; Gram-negative.

#### *Colonies*

On yeast extract mannitol agar, 1–3 mm. diameter, translucent and colorless, raised, triangular shape, tightly adhered to surface; on ferric-ammonium-citrate agar, punctiform, glistening; on crystal violet soil extract agar, a copious slimy growth developed which became yellow-gray in color.

#### *Reactions*

Broth cultures revealed a pellicle, yellow, and granular in appearance. In nutrient gelatin stabs, bright yellow and napiform liquefaction occurred after 8 days. After 10 days in litmus milk there was a slight change to alkaline, with discoloration at the base of the tubes. After 4 weeks the color had changed generally to orange, except at the surface, but with no change in consistency. In tryptone broth, moderate turbidity was produced with a slight sediment, and nitrates were reduced to nitrites.

Detailed fermentation reactions were recorded and in summary showed that acid was produced from dextrose, mannose, xylose, sucrose, maltose, glycerol, mannitol, and salicin, and the bacterium also produced moderate to heavy turbidity. In xylose a bright yellow, granular pellicle persisted but in no case was gas produced. Acetyl methyl carbinol was produced by this isolate; the methyl red test was positive, while methylene blue was reduced after 24 hours. Indole was not formed from tryptophan, but ammonia was produced from peptone. Surface growth on lead acetate agar was abundant and bright yellow, while along the inoculation stabs it was villous and colorless. No hydrogen sulphide was formed during growth on this medium. On starch agar, colonies were punctiform, with appearance, by transmitted light, of glass chips embedded in yellow slime. No diastase was produced after 8 days. On Starr's medium, colonies were flat, bright yellow with smooth surface and entire to undulate edges, but the organism showed no lipolytic activity.

It will be observed therefore that the main characteristics of isolates thus grouped are similar to those of *Flavobacterium rhenanus* (Migula) Bergey *et al.* of the family Achromobacteriaceae—an organism which does not appear to have been hitherto reported in isolations from normal and healthy plant tissue.

#### *Isolate R.4*

From both inner taproot tissue of *Trifolium pratense* and also from crushed nodules. Large, motile, Gram-positive cells, usually in chains 1.4–3  $\mu$  diameter, 3.4–9  $\mu$  length, and with round to square ends; producing cylindrical spores 1.0–1.4  $\mu$  diameter and 3  $\mu$  length and carried in slightly distended sporangia.

#### *Colonies*

On enrichment agar, flat, fawn, and granular, later developing pellucid dots; on Congo-red yeast extract agar, colonies were small, flat, with undulate edges, and they absorbed the red dye. No growth occurred on crystal violet soil extract agar but on ferric-ammonium-citrate agar colonies were circular, of entire edge, and with yellow, granular centers and gray interzones. On starch agar, flat, white colonies, reacting positively in the diastase test. There was no growth on Starr's medium.

#### *Reactions*

Methylene blue was reduced, no acetyl methyl carbinol was produced, and the methyl red test was negative. Nitrates were not reduced after 3 weeks nor was indole formed from tryptophan. In lead acetate agar, while colony growth of erose margin occurred on the surface, along the inoculation stabs it was well developed and villous, especially near the surface. No hydrogen sulphide was produced. Litmus milk was decolorized after rapid production of acid and a large, solid, buff-colored coagulum was formed, but with colorless serum. In fermentation tests, acid without gas was produced from dextrose, xylose, sucrose, maltose, and mannitol. Only slight acid reactions were observed in lactose and no acid at all in mannose or salicin.

This organism in size and many biochemical reactions resembles *Bacillus megatherium* De Bary, differing in the feature we observed of growth in salicin without production of acid.

#### *Isolate A.2*

From inner root tissue of *Trifolium subterraneum*. Short Gram-negative rod 1.4–1.7 by 1.9–2.7  $\mu$ , or coccus 0.4–0.6  $\mu$  diameter, surrounded by capsules when examined with Gram or crystal violet stain. Shadow forms common.

#### *Colonies*

On yeast extract mannitol agar, growth was copious, translucent, slimy to watery, with yellow coloration; on crystal violet soil extract agar growth flowed over entire surface, yellowish-gray in color; on Congo-red yeast extract agar, growth spreading but less copious; flat and translucent. Dilution streaks on ferric-ammonium-citrate agar, flat, gray, opaque, and glistening.

### Reactions

Gelatin liquefaction stratiform and slow; acetyl methyl carbinol and ammonia produced, and nitrates reduced to nitrites after 24 hours. On starch agar, growth copious, yellow and glistening but no diastase produced. Indole and hydrogen sulphide not formed. Slow acid production in litmus milk, with soft, buff-colored coagulate at bottom of tube only. This organism vigorously fermented the carbon compounds tested, producing acid in all media and gas in all but glycerol.

From these features this isolate would appear to be within the genus *Aerobacter*. The two type genera, *Aerobacter aerogenes* (Kruse) Beijerinck and *Aerobacter cloacae* (Jordan) Bergey *et al.* are distinguished by their actions in fermentation of glycerol and liquefaction of gelatin. The former ferments glycerol with production of acid and gas and rarely liquifies gelatin. *Aerobacter cloacae* ferments glycerol with no visible gas formation but liquifies gelatin readily. Accordingly, this isolate appeared to be identical in physiological properties with *Aerobacter cloacae*, but contrary to the above-mentioned morphological feature, Bergey *et al.* specify that this organism should be non-encapsulated.

Two other isolates were conspicuous among the bacteria isolated from within white clover (*Trifolium repens*) roots. Both were Gram-negative rods, one type being 0.3–0.5 by 0.7–1.4  $\mu$ , the other 0.3–0.6 by 1.8–2.3  $\mu$ . Both were motile, with a single polar flagellum. Detailed culture plate and fermentation reactions were recorded but thus far no tentative identification has been made. Some interest may lie in the results of tests in which these two unidentified bacteria and *Aerobacter cloacae* derived from clover tissue were inoculated into tissues of other plants. Washed slices of mature potato, onion, and carrot were prepared and held after inoculation in sterile humidity containers. The two unidentified bacteria and also the one designated above as *Aerobacter cloacae* were found to be capable of readily establishing themselves on these tissues. Each of them gave rise quickly to extensive soft rot decay in potato, carrot, and onion slices, although the nature of the rotting effects produced differed in some respects among the three bacteria used.

### Discussion

This evidence of pathogenicity (soft rot) induced by bacteria which had been passive in their original host plant impinges on that sphere of enquiry mentioned earlier, namely, the circumstances which may provoke a change from passivity to pathogenicity. In this study, there were bacteria, apparently functionless or without ill effect within clover roots, which when introduced into tissues of potato, carrot, and onion, produced extensive soft rot. It remains to be shown by work at present in progress what part is played in this development by the properties of the respective host plants and by environmental conditions experienced by the organisms following their establishment within the respective hosts.

Over all, however, the work to date seems to supply additional evidence in support of the comparatively recent finding that normal healthy plant tissue is not sterile. The studies reported herein indicate that clover root tissues carry bacteria other than the *Rhizobium* species. It is possible that as the *Rhizobium* nodule is an outgrowth of cortical root tissue, these other bacteria may become incorporated in the nodule structure. The possibility, of course, has often been referred to, including the evidence from the original description of *Bacillus radiobacter* by Beijerinck and Van Delden (1). Whilst this organism, *Agrobacterium radiobacter* and the *Bacillus concomitans* of Palacios and Bari (Bergey *et al.* (2)), can be isolated from crushed nodule preparations, it was considered by Fred, Baldwin, and McCoy (4) that evidence was not conclusive regarding the occurrence of organisms other than *Rhizobium* actually within nodule tissue.

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1. The first part of the paper is devoted to a general discussion of the problem of the origin of life. It is shown that the problem is one of the most important and most difficult in the history of science. The author discusses the various theories of the origin of life, from the spontaneous generation of life from non-living matter to the theory of the origin of life from pre-existing life. The author concludes that the most probable theory is the theory of the origin of life from pre-existing life.

2. The second part of the paper is devoted to a detailed discussion of the theory of the origin of life from pre-existing life. The author discusses the various stages of the evolution of life, from the first appearance of life to the present day. The author shows that the theory of the origin of life from pre-existing life is the only theory that can account for the complexity of life. The author concludes that the theory of the origin of life from pre-existing life is the most probable theory.

3. The third part of the paper is devoted to a discussion of the various problems that are connected with the theory of the origin of life. The author discusses the problems of the origin of the first living organisms, the origin of the first cells, the origin of the first organisms, and the origin of the first plants and animals. The author concludes that the theory of the origin of life from pre-existing life is the only theory that can account for all of these problems.

## SEASONAL VARIATION IN THE COMPOSITION OF THE BACTERIAL SOIL FLORA IN RELATION TO PLANT DEVELOPMENT<sup>1</sup>

H. G. GYLLENBERG

### Abstract

In field experiments with oats the composition of the bacterial population in the rhizosphere was found to be almost stable during the whole period of plant development from young seedlings to maturity. In the beginning of the growth season the soil flora was quite different from that of the rhizosphere. It was, however, successively changed, and became, toward the end of the season, similar in composition to the rhizosphere population. This change proceeded from the soil surface into deeper soil layers, and it can be concluded that it was due to the development of roots, and to a migration of bacteria from the rhizosphere into the soil.

### Introduction

During periods of active plant growth, three different bacterial populations can be distinguished in the soil: the population of the surfaces of plant roots; the rhizosphere flora; and the flora of the soil proper. These populations, of course, are not sharply limited from each other. In this paper the soil flora means the bacterial population of soil practically free from roots of growing plants. The root surface population mainly originates from the seed coats as demonstrated recently by Rovira (5), whereas the rhizosphere flora, as suggested by Wallace and Lochhead (7), is both of seed and of soil origin. Thus certain types of soil bacteria occur both in the rhizosphere and in the surrounding soil. This fact, however, apart from the enumeration of typical soil inhabitants in the rhizosphere, can be due also to a migration of bacteria from the rhizosphere into the soil. The purpose of the investigation, the results of which will be presented below, was to obtain further information on this point.

### Materials and Methods

Oats were selected as the test plant. The investigation concerned two experimental plots which were sowed June 1 (1955). The crop was ready for harvesting August 29. During this period samples were collected on June 1 and 17, July 15, and August 29. Each sampling series comprised three samples from each plot: (a) from the immediate soil surface, (b) from 30 cm. depth, and (c) from 50-70 cm. depth. These soil samples were taken from unsowed areas between the sowed rows. For comparison, rhizosphere samples were collected on June 17 from the soil surface, and on July 15, and August 29, from the three depths.

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Contribution from the Department of Microbiology, University of Helsinki, Helsinki, Finland.



In investigating the samples the general methods were those described earlier (1, 2, 3). Here it may suffice to note that for the characterization of the flora of each sample on a nutritional basis, some 70-100 isolates were tested.

According to our scheme (cf. (2)) the soil bacteria are classified into three main groups:

Ba—bacteria showing definite growth in the basal medium (0.8 g.  $\text{KNO}_3$ , 0.8 g.  $\text{KH}_2\text{PO}_4$ , 0.1 g.  $\text{MgSO}_4$ , 0.2 g.  $\text{NaCl}$ , 0.02 g.  $\text{FeCl}_3$ , and 2 g. glucose in 1000 ml. of distilled water, pH 6.8);

Aa—bacteria requiring amino acids as essential nutrients (the amino acids were supplied by the addition of Vitamin-free Casamino acids, Difco); and

AV—bacteria requiring both amino acids and B-vitamins as essential nutrients (the B-vitamins were supplied by the addition of a solution containing thiamine, biotin, pantothenic acid, folic acid, *p*-aminobenzoic acid, and vitamin  $\text{B}_{12}$ ).

### Results and Discussion

From the data in Table I it is obvious that the bacteria dependent on amino acids (group Aa) were most abundant in the rhizosphere samples, making up to 50-60% of the population. This agrees with the conclusions of Lochhead and Thexton (4), and also with our earlier observations concerning the rhizosphere flora of graminaceous plants in virgin soils (2, 3). Table I shows further that the composition of the rhizosphere flora remained almost unchanged during the whole experimental period. Earlier work of West and Lochhead (8) has shown that the characteristic population of the rhizosphere is found already in young seedlings. On the other hand, Starkey (6) reported that the quantitative rhizosphere effect is most pronounced in the stage of greatest vegetative development of the plant.

The results concerning the soil samples are given in Table II. These results show that in the beginning of the experimental period the composition of the soil flora was quite different from that of the rhizosphere flora. Bacteria of group Ba were distinctly predominant, and organisms of group Aa represented only an insignificant part of the population. During the course of the experimental period there occurred, however, a successive change in the composition of the soil flora. The relative abundance of Aa-group bacteria increased, whereas a corresponding decrease in the abundance of Ba-group bacteria was evident. The organisms of group AV again behaved more irregularly, although a slight increase occurred in most sampling sites toward the end of the experimental period. At the end of this period the composition of the soil flora of most sampling sites was almost similar to that of the rhizosphere flora. From the data in Table II it is evident also that the development described was already recognizable at the time of the second sampling (June 17) in the surface layer of the soil, but was not distinctly clear in the deeper layers before the third sampling (July 15). Accordingly, the change did not take place before the roots of oats had reached the corresponding depth.

TABLE I

THE RELATIVE ABUNDANCE OF THE MAIN NUTRITIONAL TYPES OF SOIL BACTERIA (GIVEN IN PER CENT OF THE TOTAL) IN THE RHIZOSPHERE SAMPLES

Depth, cm.	Time of sampling	Plot 1			Plot 2		
		Nutritional group:			Nutritional group:		
		Ba	Aa	AV	Ba	Aa	AV
0	June 17	26	62	12	11	55	34
	July 15	20	64	16	22	54	24
	Aug. 29	26	51	23	26	49	25
30	Aug. 29	10	68	22	14	58	28
50-70	Aug. 29	19	65	16	11	59	30

TABLE II

THE RELATIVE ABUNDANCE OF THE MAIN NUTRITIONAL TYPES OF SOIL BACTERIA (GIVEN IN PER CENT OF THE TOTAL) IN DIFFERENT SOILS, AT DIFFERENT DEPTHS OF SOIL, AND AT DIFFERENT TIMES OF THE SEASON

Depth, cm.	Time of sampling	Plot 1			Plot 2		
		Nutritional group:			Nutritional group:		
		Ba	Aa	AV	Ba	Aa	AV
0	June 1	58	2	40	75	6	19
	June 17	37	30	33	62	25	13
	July 15	56	24	20	52	30	18
	Aug. 29	61	23	16	20	56	24
30	June 1	92	0	8	75	11	14
	June 17	80	3	17	75	10	15
	July 15	42	36	22	55	24	21
	Aug. 29	42	56	2	26	52	22
50-70	June 1	70	6	24	86	0	14
	June 17	72	16	12	82	10	8
	July 15	54	27	19	—	—	—
	Aug. 29	24	40	36	23	48	19

In connection with the preliminary experiments reported here, it remains unexplained whether the increase in relative abundance of the Aa-group bacteria was accompanied by an accumulation of the specific nutrients (amino acids) in the soil. However, these bacteria obviously do not reach high actual numbers in the soil, and the fact that they are almost absent from cropped soils when plant growth fails (cf. data concerning the June 1 sampling, Table II) suggests that they are casual invaders of the soil. At any rate, in the soil also, the interrelationships of specific bacterial populations and specific environments are complicated, and, as also the topical results show, the "autochthonous soil flora" is not characterized by invariable stability, but rather by a dynamic state where continuous changes occur owing to influences such as plant growth.

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## EFFECT OF A SOIL MICROFLORA SELECTED BY CARBON DISULPHIDE FUMIGATION ON SURVIVAL OF ARMILLARIA MELLEA IN WOODY HOST TISSUES<sup>1</sup>

S. D. GARRETT

### Abstract

A test has been made of Bliss's hypothesis that when infected tree roots are fumigated with carbon disulphide, *Armillaria mellea* is killed not by direct fungicidal action, but through the agency of *Trichoderma viride*, which is relatively tolerant of the fumigant and becomes dominant in the treated soil. Bliss's observation that *T. viride* becomes much more abundant in soil after fumigation was confirmed, though higher dosages of carbon disulphide tended to select still more resistant fungi, such as ascospore penicillia of the *P. luteum* series. When small woody inocula of *A. mellea* had been incubated for 3 weeks in soil that had been previously fumigated with carbon disulphide at the rate of 828 p.p.m. and then kept for 3 weeks before use, 30% of the inoculum segment-ends failed to produce rhizomorphs when placed in tubes of fresh soil for a viability test. Nevertheless, this indirect effect of soil fumigation cannot wholly account for the total loss of viability when inocula of *A. mellea* are directly fumigated in soil. It seems, therefore, that carbon disulphide must directly damage at least the peripheral mycelium of *A. mellea* in the woody host tissues, and so facilitate invasion by *T. viride* and/or other fungi. With this additional postulate, Bliss's hypothesis seems to account well for all the observed facts.

### Introduction

It is now 25 years since Weindling (19) published his first paper on *Trichoderma viride* as a parasite of other soil fungi. The widespread attention commanded by this paper was sustained by further communications from the same author and his collaborators (20, 21, 22, 23, 24). The "lethal principle", as it was first designated by Weindling (20), thus excreted by *T. viride*, was subsequently further characterized and named "gliotoxin" by Weindling and Emerson (23). A second antibiotic produced by *T. viride*, more potent but also more unstable, was subsequently discovered and characterized under the name of "viridin" by Brian and McGowan (4, 5). References to subsequent work on these two antibiotics are given in the compilation by Brian (3).

Since the first demonstration by Weindling, many other workers have shown that *T. viride* can exercise both fungicidal and fungistatic effects upon other fungi grown in juxtaposition with it upon an agar plate. By suitable inoculation techniques, control of soil-borne fungal pathogens of crop plants by *T. viride* has been demonstrated in sterile soil. The limitations of such biological control in *unsterilized* soil, however, were early demonstrated by Weindling and Fawcett (24), who showed that protection of citrus seedlings from infection by *Rhizoctonia solani*, apparently through the agency of *T. viride*, could be achieved by soil acidification. When conditions were thus made suitable, by soil acidification, for possible antibiotic activity of *T. viride*, then inoculation of the soil with this fungus, so as to increase its population

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above the natural level, sometimes gave increased control of *R. solani*; in the absence of such acidification, however, even heavy inoculations with *T. viride* were useless for biological control.

Further evidence for the natural control of a root disease fungus by *T. viride*, but only in acid soils, has been produced by Rishbeth (14, 15), who has found that under such conditions the advance of *Fomes annosus* along pine tree roots is largely confined to internal infection. This contrasts strongly with the rapid ectotrophic advance of the fungus along pine roots in alkaline soils. Rishbeth has demonstrated that inhibition of ectotrophic growth by *F. annosus* in acid soils is most likely to be an effect of the root surface flora, and he has produced a variety of evidence in support of his hypothesis that *T. viride* is one of the chief root surface fungi contributing to this effect.

The first demonstration that significant differences in root surface and rhizosphere microfloras might be associated with differences in resistance of higher plants to root diseases was, however, published 10 years earlier by Lochhead and his research associates, in the course of a fundamental and far-reaching study of the rhizosphere in general. The researches thus directed by Lochhead are not merely the most substantial contribution towards our understanding of the rhizosphere since its discovery by Hiltner (10); they have also provided an entirely new approach, through the technique of nutritional grouping, to the recognition and study of bacteria in the soil.

In the course of this general study, Lochhead, Timonin, and West (11) reported significant differences in the rhizosphere microfloras of varieties of flax susceptible and resistant, respectively, to wilt (*Fusarium oxysporum lini*), and of varieties of tobacco susceptible and resistant to black root rot (*Thielaviopsis basicola*). This lead was followed further by one of Lochhead's associates, Timonin (16), in a brilliant analysis of the factors underlying characteristic differences in the fungal constituents of the rhizosphere in two varieties of flax, susceptible and resistant, respectively, to wilt. For this purpose, Timonin constructed root models in the form of collodion membrane sacs filled with used nutrient solutions, in which the susceptible and resistant varieties of flax, respectively, had been growing for 25 days. When these root models were placed in soil, the contained nutrient solutions diffused outwards, producing artificial rhizospheres similar, in their differences between susceptible and resistant varieties of flax, to the natural ones. Particular interest is attached here to Timonin's finding that *T. viride* was more abundant in the rhizosphere of the wilt-resistant variety (and in the artificial rhizosphere of the analogous root model). Following up the earlier finding of Reynolds (13) that resistance of different varieties to flax wilt could be correlated with hydrocyanic acid content of the plant tissues, Timonin demonstrated that the preponderance of *T. viride* in the rhizosphere of the wilt-resistant variety could be ascribed to its remarkable tolerance of cyanide. Nevertheless, it is important to note here that Timonin quite correctly forbore to postulate any causal connection between the occurrence of *T. viride* in the rhizosphere and the resistance of the flax variety to wilt. The simpler hypothesis that

hydrocyanic acid (or cyanide) is the factor directly responsible for wilt resistance is equally admissible; nor are the two hypotheses mutually exclusive.

Whereas we now have considerable evidence for the view that *T. viride* plays some part, and perhaps an important part, in the natural biological control of some soil-borne diseases of plants, we are still as far as ever from a method for further practical exploitation of this effect. Great interest has therefore been aroused by a possible new approach to the problem suggested by Bliss (2), who postulated that the killing of *A. mellea* in infected tree roots by soil fumigation with carbon disulphide was due not to direct fungicidal action, but to an indirect effect. Bliss had noted that whenever *A. mellea* failed to develop from fragments of infected wood from fumigated roots plated-out on agar, colonies of *T. viride* grew out instead. He therefore suggested that *T. viride* was directly responsible for the death of *A. mellea*, and that the effective mechanism of carbon disulphide fumigation was to increase the soil population of *T. viride* to such an extent that it was able to break through the protective outer wall (seen as a dark "zone-line" in sections of infected wood) of the pseudosclerotium of *A. mellea* in infected roots (6). The normal population of *T. viride* present in untreated soil was considered to be inadequate to breach the outer wall of the pseudosclerotium in this way. Bliss's hypothesis is thus based upon the fact that *T. viride* is more resistant to the fungicidal effect of carbon disulphide than are most other soil fungi, just as Timonin (16) had observed it to be more tolerant than most fungi of cyanide. I have discussed elsewhere (8) the remarkable resistance of *T. viride* to a variety of fungicides, including antibiotics. It is also of interest to note a very similar general suggestion, but with much less evidence in support, made independently by Altson (1).

Bliss supported his hypothesis by three kinds of evidence: (1) when the viability of *A. mellea* was tested by plating-out fragments of tissue from the interior of fumigated roots, death of the fungus was sometimes not complete until some 24 days after fumigation; (2) when roots colonized by *A. mellea* in pure culture were fumigated in sterile (autoclaved) soil, the fungus was not killed; and (3) *A. mellea* was killed if infected roots were buried in a pure culture of *T. viride* grown on autoclave-sterilized soil.

The evidence thus put forward by Bliss, although remarkably convincing at first sight, can be criticized in several ways. Firstly, Bliss's technique for determining viability of *A. mellea* in infected root fragments by plating-out on agar can sometimes give misleading results; most specialized root-infecting fungi are characterized by a low degree of competitive saprophytic ability (8), and are likely to be suppressed by associated saprophytes in competition for colonization of nutrient agar. Secondly, the survival of *A. mellea* in roots fumigated in autoclaved soil does not prove that fumigation had no direct effect on the fungus; to establish this, a more sensitive and quantitative technique than Bliss's method of tissue culturing from small samples would be necessary. Thirdly, the demonstration that *A. mellea* can be killed by burying infected roots in a pure culture of *T. viride* grown on sterilized soil is



not now so surprising, nor so relevant as evidence in support of Bliss's hypothesis, as would formerly have been supposed. This point can most simply be explained by reference to the concept of *inoculum potential*, which I have defined and discussed elsewhere (8). There is now ample evidence that the inoculum potential of a microorganism determines whether or not it will be able, if a parasite, to establish a successful, progressive infection in its host; in addition, inoculum potential appears sometimes to be of critical importance in the saprophytic colonization of dead substrates. Now in pure culture on sterilized soil, the inoculum potential of a fungus is at its maximum possible level for this substrate, in so far as all the available nutrients in the soil can be assimilated in the absence of competitors. But a fungus that we describe as "dominant" in a fumigated soil, because it appears according to our criteria to be more obviously abundant than others, is sharing the habitat with other, but less abundant, fungi, and also with a variety of other microorganisms, including bacteria, which may even become more numerous, if fumigant-resistant, than in untreated soil. The inoculum potential of a dominant fungus in a natural or seminatural community of soil microorganisms is therefore certain to be lower, and probably very much lower, than that of the same fungus grown in pure culture on completely sterilized soil. The high inoculum potential of a pure culture of *T. viride* on sterilized soil is likely to give it a decisive advantage in its "attack" upon *A. mellea*; if this attack is brought about by the excretion of gliotoxin and/or viridin, then the concentration of these antibiotics is likely to be at a maximum in sterilized soil, as has been demonstrated by a number of investigators for a variety of antibiotics (8).

Despite these criticisms, the hypothesis proposed by Bliss is worthy of most careful consideration and further testing. The investigation to be reported below forms the second part of this testing of Bliss's hypothesis. The first part has already been published by my former research associate, Dr. E. Evans (7). Working with Kettering loam, a soil that has been much studied in the Cambridge Botany School, Evans found that treatment with carbon disulphide, at the rate of 2 ml. per kg. air-dry soil for a period of 24 hours, subsequently produced a dominance of *T. viride* on Warcup soil plates (17) made from the fumigated soil. With doses of 4 ml. and upwards of carbon disulphide, however, *T. viride* was replaced on the soil plates by still more resistant ascosporic ascomycetes, mainly *Aspergillus fischeri* and yellow penicillia of the *Penicillium luteum* series. When tested for tolerance to carbon disulphide in pure culture, these ascosporic ascomycetes were markedly more resistant than was *T. viride*, which in turn was more resistant than the other soil fungi tested.

My chief asset for the undertaking of this investigation has been the possession of a sensitive technique, developed for other purposes (9), for estimating not merely the viability, but also the vigor and total resources, of a mycelial mass of *A. mellea* in a corpus of woody tissue infected or colonized by the fungus. By this technique, small, woody inocula, originally colonized by *A. mellea* in pure culture, and later subjected to the various treatments to



be tested, are buried under standardized conditions in columns of natural, unsterilized soil set up in open-ended glass tubes. Rhizomorphs develop and grow out into the soil from each end of the wood segment constituting the inoculum; rhizomorph growth is estimated by weekly measurements, and weekly growth increments are calculated. The trend of growth increments thus obtained affords a sensitive index of the physiological condition of the mycelial mass of *A. mellea* in the inoculum to be tested.

### Materials and Methods

The soil employed in this work (Kettering loam) was the same as that used in a preceding study (7) of the effect of carbon disulphide and of formalin on the fungus flora. In texture, the soil is a medium loam, and the pH ranges from 5 to 6. Organic matter and total nitrogen are somewhat high, as the soil is under grass; available potash and phosphate are rather low. The moisture-holding capacity (hereafter abbreviated to "m.h.c.") varies around 66 ml. water per 100 g. air-dry soil, and this reflects the rather high organic content. The soil was collected as required from a dump at the Botany School Field Station, and was passed through a  $\frac{1}{4}$  in. sieve; for Experiment 1, it was further passed through a fine sieve (14 mesh per in.).

Fumigation of the soil was carried out at 25° C.; screw-topped jars of square cross section and capacity 2400 ml. were used as containers. To prevent escape of the fumigant, tops of the jars were tightly screwed down and then bound with "Sellotape". Each fumigation jar received some 1800 g. soil at a moisture content near 40% m.h.c.; the low moisture content was chosen to facilitate dispersal of the fumigant. Carbon disulphide was injected into the soil at the mid-point of each jar; if woody inocula permeated by *A. mellea*, or uninoculated woody "baits" for colonization by soil fungi, had to be fumigated in the soil, then they were disposed equidistantly around the point of injection. Details of dosages and times of fumigation are given under individual experiments.

Particulars of preparation of woody inocula of *A. mellea* and of the technique used for measurement and calculation of rhizomorph growth rate are given in a previous paper (9), but will be briefly recapitulated. For the preparation of inocula, shoots not exceeding 1.75 cm. diameter were cut from pollarded willows (*Salix alba*) and sawn into segments of length 2.5 cm., which were then weighed; segments of the required weight were subsequently selected from these. Sufficient segments to occupy the bottom of a 250 ml. conical flask were covered with maize meal-sand culture medium (in proportion 100 g. sand : 3 g. maize meal : 15 ml. water). After being autoclaved for 1 hour at  $1\frac{1}{2}$  atm. pressure, flasks were inoculated with *A. mellea* and then incubated for  $2\frac{1}{2}$  months at 25° C. Thereafter they were stored in the dark for at least another  $2\frac{1}{2}$  months.

To secure standard and most favorable conditions for the final test of rhizomorph-producing capacity in the variously treated inoculum segments, the soil used in the glass tubes for rhizomorph growth measurement was not

Kettering loam, but a light loam from my own Cambridge garden, as used in the earlier study (9). Each inoculum segment to be tested was buried at the mid-point of an open-ended glass tube, of 1.9 cm. internal diameter and length 25 cm. Air-dry soil was filled into each tube in short sections (4-5 cm.), which were then brought to a moisture content of 60% m.h.c. with distilled water from a graduated pipette. When set up, each tube was capped at either end with moisture-proof, lacquered "Cellophane". The soil tubes thus prepared were incubated for rhizomorph development at 25° C.

Visible production of rhizomorphs from either end of most inoculum segments usually occurred within 10-14 days at 25° C. Thereafter, weekly measurements of the maximum extent of rhizomorph growth from either end of the inoculum segment (i.e. up or down, as the tubes were incubated vertically in baskets) were made at weekly intervals. Because rhizomorphs could be seen only when they impinged upon the glass wall of the tube, the maximum extent of rhizomorph growth thus measured was likely to be always somewhat less than the real extent.

From the data thus obtained, a series of weekly growth increments of rhizomorphs from either end of each inoculum segment was calculated. This method of treating the data eliminated the variation that would otherwise have been caused through the fact that rhizomorph production started from different inoculum segments (and even from the two ends of a single inoculum segment) at different times. The first weekly growth increment to be recorded for any inoculum segment-end might represent less than a full week's growth; it was excluded on this account only if it was less, by more than 25%, than the second weekly growth increment for the same inoculum segment-end.

For interpretation of the data to be presented below, the main conclusions from my previous study of rhizomorph growth must be given. Initial growth rate was found to depend on the size of the inoculum. A subsequent progressive decline in growth rate was attributed to: (1) gradual consumption of nutrient reserves in the inoculum through fungal respiration and growth, (2) increasing competition for available nutrients between the main growing apex of the rhizomorph and its subordinate branch apices.

## Experimental

### *Experiment 1*

In this experiment, the dosage of carbon disulphide was approximately equated with that used by Bliss (2) in the three experiments described in the final section of his paper, under the heading "The role of *Trichoderma* in soil disinfection". Bliss fumigated his jars of soil and/or infected roots by placing them in a large steel container, into which was injected carbon disulphide at the rate of 59.1 ml. per 9 cu. ft. space; this represents a concentration of 232 p.p.m. in air (in Bliss's paper, the concentration is wrongly given as 2.32 p.p.m.). This dose is equivalent to 1 ml. carbon disulphide per 4312 ml. space, so that for a jar of 2400 ml. capacity, as used here, 0.56 ml. carbon disulphide would be required for exact equivalence. For convenience

in pipetting, the actual dose applied in this first experiment was 0.5 ml., representing 207 p.p.m. carbon disulphide. The period of fumigation was shorter—72 hours instead of 7 days, as used by Bliss.

The inoculum segments immediately available for this experiment happened to be of three sizes, but all were of the same length, 2.5 cm.; fresh weights before inoculation were near 4, 2, and 1 g., respectively. After inoculation with *A. mellea* in pure culture, the segments had been incubated for 2½ months at 25° C., and thereafter for another 6½ months at laboratory temperature. To each of the six experimental treatments to be described below, five inoculum segments of each size were allotted; this gave a replication of 10 rhizomorph growth measurements for each size of inoculum, or a total of 30 measurements for each experimental series.

The experiment was designed to analyze the total effect of fumigation on *A. mellea* into its postulated two components: (1) a *direct* effect of the fumigant upon the fungus, (2) an *indirect* effect, through the changed microflora arising as a result of soil fumigation. After completion of the fumigation treatments, inoculum segments were incubated for a period of 3 weeks at laboratory temperature under the soil conditions described below. They were then removed from the Kettering loam soil, brushed clean, and set up in growth tubes of Cambridge garden soil as described above, for weekly recording of rhizomorph growth.

*Treatment 1. Normal fumigation (direct + indirect effects).*—Inoculum segments were fumigated for 72 hours at 25° C. in Kettering loam at a moisture content of 40% m.h.c., and then incubated in the fumigated soil (with cover of jar removed) for 3 weeks at laboratory temperature.

*Treatment 2. Direct effect only.*—Inoculum segments were fumigated in steam-sterilized soil (steamed at 1–8 lb. pressure for 1 hour on 3 consecutive days). After fumigation, the cover of the jar was unscrewed, to facilitate dispersal of the fumigant, but left lying loosely on the neck, as a protection against recontamination.

*Treatment 3. Direct effect followed by burial in unfumigated soil.*—Inoculum segments were fumigated in steam-sterilized soil, and then removed, brushed clean, and kept for 24 hours in a flask through which was passed a rapid stream of moist air, to remove all trace of the fumigant. Inoculum segments were then reburied in unfumigated soil, and incubated therein for 3 weeks.

*Treatment 4. Direct effect reconstituted with full indirect effect.*—Treatment as for treatment 3, above, but inoculum segments were reburied after fumigation and incubated for 3 weeks in soil that had received the standard fumigation, followed by 2 weeks' storage at laboratory temperature.

*Treatment 5. Indirect effect only.*—Inoculum segments taken straight from the culture flasks (and brushed free of adhering maize meal–sand culture medium) were buried and incubated for 3 weeks in soil that had received the standard fumigation, followed by 2 weeks' storage at laboratory temperature.

*Treatment 6. No treatment (control).*—Inoculum segments were incubated for 3 weeks in untreated soil.

The results of this experiment can be quickly described. In every treatment in which inoculum segments were fumigated (i.e. Nos. 1-4), *A. mellea* was apparently killed. This assumption is based on the fact that not a vestige of rhizomorph growth appeared from any inoculum segment from these four series over the observation period of 60 days, whereas rhizomorphs were put out by segments from treatments 5 and 6 within 11-18 days. The results from treatment 2 (fumigated in sterilized soil and incubated *in situ*) are not so conclusive as they should have been, because in spite of precautions during transfer of inoculum segments from the pure culture flasks to the jar of sterilized soil, two spreading colonies of *T. viride* appeared on the surface of the soil during the 3 week incubation period after fumigation. When the inoculum segments were removed from the soil prior to setting in the soil tubes for testing, some of them were found to be covered by a vigorous growth of green, sporulating *T. viride*. It is evident, therefore, that air-borne contamination of the segments must have occurred during transfer from pure culture flasks to sterilized soil, and that the fumigation must have killed all air-borne contaminants except *T. viride*. Nevertheless, the growth of *T. viride* on the segments that became colonized was so profuse that segments not thus visibly contaminated may be presumed to have been sterile. Since these latter segments also failed to produce any rhizomorphs, it can be assumed that the direct effect of fumigation was sufficient to kill *A. mellea*. It is also relevant to note the complete kill of *A. mellea* by treatment 3, in which fumigation in sterile soil was followed by burial in untreated soil with an unchanged microflora, in which no indirect effect of fumigation could possibly have been involved.

We may finally consider the results of treatments 5 and 6, in which inoculum segments buried for 3 weeks in previously fumigated and then incubated soil are compared with segments buried for the same period in untreated soil. There was no significant difference between the two series either in the time at which rhizomorphs commenced growth, or in the very small proportion of inoculum segment-ends that failed to produce rhizomorphs over the observation period of 60 days. Weekly growth increments of rhizomorphs produced by the two sets of inoculum segments are given in Table I.

No statistical examination of the data in Table I has been made, as inspection is sufficient to show that the slight reduction in rhizomorph growth increments for the inoculum segments incubated in previously fumigated soil would not prove to be significant.

TABLE I  
MEAN WEEKLY GROWTH INCREMENTS OF RHIZOMORPHS (MM.)

	Weekly growth periods					Mean
	1	2	3	4	5	
Unfumigated soil	12	12	12	8	8	10
Fumigated soil	12	12	8	9	5	9

### Experiment 2

Although a comparison of treatments 5 and 6 in the preceding experiment had afforded no evidence for an indirect microbiological effect of soil fumigation on the viability or vigor of *A. mellea*, it was deemed necessary to make a further test of this possibility, using a wider range of doses and dosage times for the fumigation and determining the dominant fungi developing on wood "baits", similar to the inoculum segments but not inoculated, to be included in the soils to be fumigated. Three doses of carbon disulphide (0.5, 1, and 2 ml.) were therefore factorially combined with four dosage times (24, 48, 72, and 96 hours), making 12 treatment series in all. As a 13th treatment, unfumigated soil was included as a control. The Kettering loam soil was at a moisture content of 45% m.h.c. at time of collection, when it was passed through a  $\frac{1}{4}$  in. sieve only. For determination of dominant fungi likely to colonize the inoculum segments, 20 fresh willow shoot segments (*not* inoculated with *A. mellea*), of size  $1.75 \times 2.5$  cm., were buried in each soil jar before fumigation; these will be referred to as "bait segments". After fumigation, covers of jars were removed to permit dispersal of the carbon disulphide, and the jars were incubated at laboratory temperature for 22 days after removal of covers from the 96 hour series.

During the 3rd week of this incubation period, green plaques of sporulating *T. viride* appeared between the soil and the glass walls of the fumigation jars; the relative abundance of *T. viride* was visually rated (Table II).

It must be noted, however, that even the strongest development of *T. viride* recorded in Table II (++++) for the 1 ml., 96 hour series) was thin and scattered by comparison with the uniform and heavy development of sporulating mycelium to be seen on a pure culture of this fungus growing on completely sterilized soil. A similar sporulation of *T. viride* at the soil surface after treatment with formalin was observed by Warcup (18).

This spontaneous development of *T. viride* on the fumigated soils was used as a guide to selection of four of the treatment series for further study; the four treatments selected were the three doses, 0.5, 1, and 2 ml., of carbon disulphide in the 96 hour series, together with the unfumigated control soil.

After this 22 days' incubation of the fumigated soils had been completed, the 20 bait segments were removed from each of the four selected jars. It was then noted that the cut ends of the bait segments from the 2 ml., 96 hour

TABLE II  
RELATIVE ABUNDANCE OF *Trichoderma viride* DEVELOPING AT SOIL/GLASS  
INTERFACE AFTER FUMIGATION

	Treatment times, hr.			
	24	48	72	96
0.5 ml. carbon disulphide	—	+	+	+
1 ml. carbon disulphide	+	++	+++	++++
2 ml. carbon disulphide	++	—	—	+



series were covered with a bright yellow growth of mold. Examination of the bait segments from the nine treatments to be discarded revealed a similar and almost continuous development of yellow mold on the cut ends of the bait segments in the 2 ml., 72 hour series as well. The fungus proved to be a *Penicillium* of the *P. luteum* series (12); numerous bright yellow globose perithecia (later turning orange-yellow) were enmeshed in mycelium of the same color. This confirmed the observation of Evans (7), already noted above, that higher doses of carbon disulphide produced a dominance not of *T. viride*, but of ascosporic ascomycetes, including *P. luteum*. I should add, however, that the small number of perithecia that I cultured from, and examined microscopically, appeared to be those of *P. wortmanni* (in the *P. luteum* series); this identification was confirmed by Prof. K. B. Raper. The sample was not large enough, however, to be conclusively representative and it is quite possible that several species in the *P. luteum* series were present.

The 20 bait segments from each of the three selected treatments, together with those from the unfumigated control soil, were then soaked in water for 10 minutes, washed clean, allowed to drain, and placed on wads of wet filter paper in jars covered with moisture-proof "Cellophane". After 11 days' incubation at laboratory temperature, an assessment was made of the fungus cover on the upper ends of the segments, which had been stood upright in the moist chambers.

*2 ml., 96 hour series.*—Although the yellow penicillia had constituted the sole visible fungus cover over the ends of the segments when these were removed from the soil, yet on the washed and incubated segments, *T. viride* was codominant with the penicillia. The mean percentage cover of both the yellow penicillia (*P. luteum* series) and of *T. viride* was estimated at 33%, leaving 33% of the area uncovered by mycelium.

*1 ml., 96 hour series.*—*Chaetomium* sp. (probably *C. olivaceum*) was dominant on 8/20 bait segments. *T. viride* was dominant on 3/20 segments and occurred as macroscopic colonies on another 5/20. Penicillia of the *P. luteum* series occurred on 4/20 segments.

*0.5 ml. series and unfumigated control.*—Various dark fungi had covered the cut ends of the bait segments; a species of *Stysanus* was prominent.

The conclusion from the earlier observations on the undisturbed soil and on the bait segments, before washing and further incubation, had been that *T. viride* was the dominant fungus developing in the 1 ml., 96 hour series, and that yellow penicillia of the *P. luteum* series were dominant in the 2 ml., 96 hour series. This agreed with the earlier study by Evans (7), using Warcup soil plates for sampling and identification of the soil fungi. This conclusion is at variance, however, with the observations on the bait segments after washing and further incubation, when *T. viride* was found to be codominant with the yellow penicillia. The most likely explanation for this discrepancy is that the population of *T. viride* was more severely reduced by the 2 ml., 96 hour treatment than was that of the yellow penicillia, but that it gradually increased during the post fumigation period of incubation.

After removal from the fumigation jars at the end of the 22 day incubation period, the four soils selected for further study were each well mixed and then disposed in four equal subsamples of each soil into 2 lb. jam jars (for greater convenience of handling). In each small jar were buried five inoculum segments of *A. mellea*, giving 20 inoculum segments for each soil series. The inoculum segments (all of 4 g. fresh weight before inoculation) had been incubated in the culture flasks for 2½ months at 25° C., and then for another 2½ months at laboratory temperature.

The 16 jars of soil, with their contained inoculum segments, were then incubated for 23 days, at a laboratory temperature fluctuating between 19° and 25° C., but nearer to 25° C. for the greater part of the period. As antagonistic effects of *T. viride* on some other fungi are greatest at or above 20° C. (14, 15), every chance was given by this period and temperature of incubation for *T. viride* to replace *A. mellea* in the inoculum segments. At the end of the incubation period, inoculum segments were removed from the treated soils and brushed clean; each one was then set up at the mid-point of a growth tube filled with Cambridge garden soil, and then all tubes were incubated at 25° C. During the manipulation, opportunity was taken to examine the inoculum segments under the binocular dissecting microscope for surface colonies of *T. viride*. The segments from the 2 ml. series carried the most colonies and those from the unfumigated soil the least; there was little difference between segments from the 0.5 and 1 ml. series.

The results of this experiment are assessed in terms of the behavior of the 40 inoculum segment-ends belonging to the 20 segments buried in each of the four soil series. In Table III is given the number of segment-ends developing rhizomorphs over the 75 day period of observation. In Table IV, mean weekly growth increments of rhizomorphs are given, assessed from those segment-ends that produced rhizomorphs, i.e. those that produced none are excluded from calculation of the means.

Table III shows that 12/40 (30%) segment-ends from the soil previously fumigated with 2 ml. carbon disulphide failed to produce rhizomorphs during the 75 day period of observation; *A. mellea* can therefore be considered as non-viable in at least part of the inoculum segments to which these ends belonged. Eight of the 12 segment-ends were paired as opposite ends of the

TABLE III  
NUMBER OF INOCULUM SEGMENT-ENDS PRODUCING RHIZOMORPHS

	Observation periods (in days from beginning)					
	12	19	26	33	40	75
No fumigation of soil	34	38	39	39	39	39
0.5 ml. carbon disulphide	29	39	40	—	—	—
1 ml. carbon disulphide	31	38	38	38	39	39
2 ml. carbon disulphide	16	23	26	26	27	28



TABLE IV  
MEAN WEEKLY GROWTH INCREMENTS OF RHIZOMORPHS (MM.)

	Weekly growth periods					Mean ( $\pm 0.53$ )
	1	2	3	4	5	
No fumigation of soil	20	14	14	15	10	15
0.5 ml. carbon disulphide	22	15	14	9	5	13
1 ml. carbon disulphide	22	14	11	9	6	12
2 ml. carbon disulphide	19	15	11	9	8	12
Mean ( $\pm 0.59$ )	21	15	13	11	7	

NOTE: Standard error for individual treatment means = 1.18.

same segment, so that *A. mellea* appeared to be completely non-viable in 4/20 (20%) of whole inoculum segments. It will be remembered that in this soil, *T. viride* showed the most abundant development, firstly on the washed and incubated wood baits, and later on the surface of the inoculum segments themselves, when these were removed from the soil. By contrast, loss of viability by *A. mellea* was restricted to 1/40 segment-ends from the untreated soil and from that treated with 1 ml. carbon disulphide, and there was no loss of viability by *A. mellea* in the segment-ends from the soil treated with 0.5 ml. carbon disulphide.

In Table IV are given rhizomorph growth data for the inoculum segment-ends that did produce rhizomorphs. An analysis of variance performed on these data shows that the effect of previous incubation of inoculum segments in the variously treated soils, though not large, is statistically significant ( $P = 0.05$ ). The interaction between incubation treatments and weekly growth increments of rhizomorphs is highly significant ( $P = 0.001$ ). This can be explained by inspection of the trends in weekly growth increments; there is no difference amongst the four series for the first two increments, but thereafter growth increments for the three series of inoculum segments buried in fumigated soils decrease more rapidly than those for segments incubated in the unfumigated control soil.

### Discussion

It is now necessary to decide how far Bliss's hypothesis can be reconciled with the new observations reported above. The results of experiment 1 showed that fumigation with 0.5 ml. carbon disulphide (207 p.p.m.) for 72 hours in sterile soil was sufficient to kill *A. mellea* in small inoculum segments (1.75  $\times$  2.5 cm.) by direct fungicidal action. Examination of the wood baits fumigated in the soils of experiment 2 showed that quadruple this dose (828 p.p.m.) was required to kill the majority of saprophytic soil fungi, and thereby to produce a maximum dominance of the fumigant-resistant *T. viride*. If the carbon disulphide has equal access both to *A. mellea* and to *T. viride*, therefore,

a smaller dose is required to kill *A. mellea* by direct fungicidal action than that needed to produce soil dominance by *T. viride*. This conclusion, however, does not invalidate Bliss's hypothesis, which postulates that a dosage of carbon disulphide insufficient to penetrate to the center of a sizeable infected root, and thus insufficient to kill internal mycelium of *A. mellea* by direct action, may yet be sufficient to produce a local dominance of *T. viride* at the root surface, where the concentration of the fumigant is higher.

If Bliss's hypothesis were absolutely correct in its original form, then we should expect that *A. mellea* would be killed as effectively by burying inoculum segments in previously fumigated soil that had developed a new fungus flora dominated by *T. viride* as by direct fumigation of the inoculum segments themselves. Tables III and IV show that this expectation has not been fully realized: 30% of inoculum segment-ends from the soil fumigated with 2 ml. carbon disulphide, which had eventually developed the highest population of *T. viride* on wood baits and again on inoculum segments, failed to produce rhizomorphs; amongst the 70% of inoculum segment-ends that did do so, rhizomorph growth increments were not much less than those for inoculum segments incubated in untreated soil. It thus seems that the indirect effect of soil fumigation, although by no means negligible, is insufficient to account for the 100% kill of *A. mellea* obtained by fumigation of the inoculum segments themselves, with only one quarter the dose of carbon disulphide.

We can therefore conclude that carbon disulphide must exert some direct fungicidal action upon *A. mellea*, to explain the results obtained in these experiments. Nevertheless, it is impossible to account for Bliss's observations by supposing that direct fungicidal action was solely responsible for the death of *A. mellea* in the sections of fairly large infected roots that he used in his fumigation trials, because he repeatedly observed that loss of viability by *A. mellea* was not complete until some 3-4 weeks after fumigation. It is tempting to suggest that carbon disulphide directly damages the outer wall of the pseudosclerotium of *A. mellea* immersed in the woody tissues, and thereby permits invasion of the interior by *T. viride* and/or other fungi. Even if we assume, however, that the pseudosclerotium wall (or "zone-line") is an effective barrier against invasion by other fungi, it must still be remembered that complete zone-lines are not always formed at the exposed ends of infected root sections; complete zone-lines, or their equivalent as surface xylostroma (6), were only very rarely formed across the cut ends of the inoculum segments used in this investigation. Rishbeth (15) concluded that gradual but not too severe desiccation promoted zone-line formation by *F. annosus*; it seems probable that the cut ends of the inoculum segments of *A. mellea* in the culture flasks remained too moist for zone-lines to form, though conspicuous zone-lines were eventually produced in the surrounding matrix of maize meal-sand culture medium as it dried out. From the evidence of this investigation, therefore, it seems that *A. mellea* can survive in infected wood, at least under normal conditions, without the protection of zone-lines.

This is not surprising, in view of the fact that root-infecting fungi commonly survive even in the unprotected infected tissues of herbaceous plants for periods of a year or more (8).

A possible mechanism whereby peripheral and limited action of carbon disulphide could start off a progressive invasion of infected roots by *T. viride* may be postulated as follows. It may be assumed that a fumigant-selected population of *T. viride* can invade without difficulty the cut ends of fumigated root sections in which *A. mellea* has been killed or fatally injured by direct action of carbon disulphide. During this process of initial invasion, *T. viride* must greatly increase its inoculum potential, and provide itself with a food base from which a successful, progressive invasion of the remainder of the infected root can be initiated. On this hypothesis, the action of carbon disulphide may be envisaged as upsetting the normal equilibrium between *A. mellea* in the infected root and *T. viride* at the root/soil interface; this hypothesis differs from that of Bliss inasmuch as a direct effect of the fumigant upon *A. mellea* as well as upon the soil fungus population is postulated. The initial momentum of invasion thus gained by *T. viride* is likely to carry it at least for some distance into the infected root, but the extent of the invasion thus initiated requires investigation. The invasion of roots infected by *F. annosus* from woody inocula of *T. viride* has been studied by Rishbeth (14, 15); invasion by *T. viride* was most successful at the highest temperature tested (15° C.), and could be inversely correlated with the freshness and vigor of the *F. annosus* mycelium, i.e. old and rotted infected roots were more susceptible to invasion than recently infected ones.

Further experiments are now needed to test this modification of Bliss's hypothesis, but the available evidence on the connection between inoculum potential of *T. viride* and its capacity to invade woody tissues already occupied by *A. mellea* may be recapitulated. In experiment 2, incubation of inoculum segments for 3 weeks in fumigated soil (2 ml. carbon disulphide) having the maximum observed population of *T. viride* resulted in a loss of viability by *A. mellea* not exceeding 30%. But by incubating infected root sections in a pure culture of *T. viride* on autoclaved soil, which would have had a much higher inoculum potential than a fumigant-augmented population of this fungus, Bliss obtained a 100% kill of *A. mellea*.

Whatever the eventual outcome of this investigation, there can be no doubt as to the great value both of Bliss's observations and of his original conception, which has opened up a new vista of possibilities for control of microbiological equilibria in the soil.\*

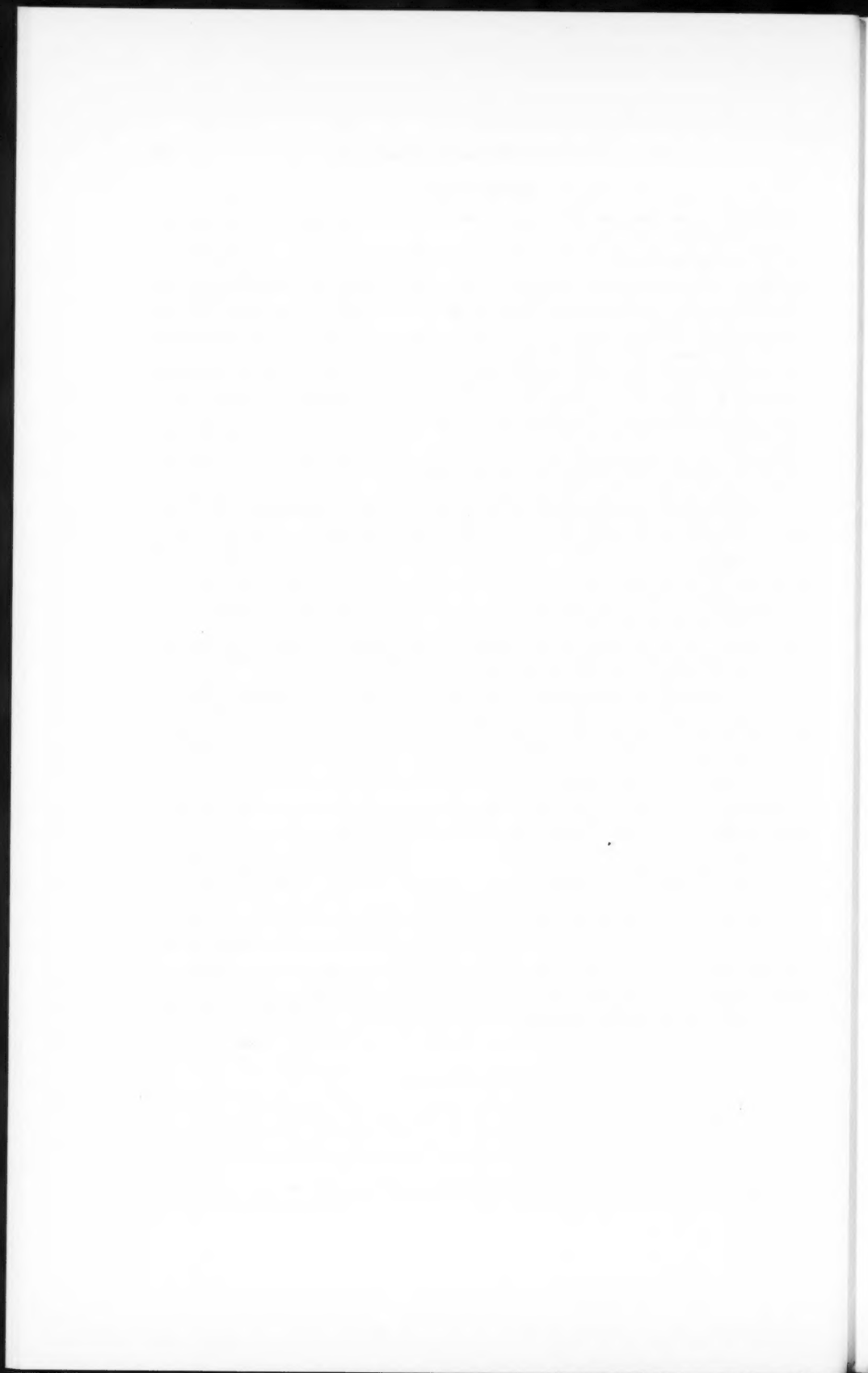
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\*The late Dr. D. E. Bliss died on October 4, 1951, only a few months after the publication of the paper discussed here.

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## DECOMPOSITION OF CHLORO-SUBSTITUTED ALIPHATIC ACIDS BY SOIL BACTERIA<sup>1</sup>

H. L. JENSEN

### Abstract

Three groups of bacteria capable of decomposing chloro-substituted aliphatic acids were isolated from soil by means of selective media. A group of *Pseudomonas*-like bacteria (A) decomposed monochloroacetate (and monobromoacetate) readily in media with yeast extract, peptone, or amino acids. They also decomposed  $\alpha$ -monochloropropionate with moderate vigor, but had little effect on dichloroacetate and -propionate, and none on trichloroacetate. A non-sporeforming bacterium of uncertain taxonomic position (B) was able to decompose trichloroacetate in media containing soil extract or vitamin B<sub>12</sub>, and also in basal medium when associated with vitamin B<sub>12</sub>-producing strains of *Streptomyces*. Dichloroacetate was only slightly attacked, and monochloroacetate and  $\alpha$ -dichloropropionate not at all. A group of bacteria (C) apparently belonging to *Agrobacterium* decomposed  $\alpha$ -dichloropropionate and dichloroacetate, but was less active towards  $\alpha$ -monochloropropionate, and did not attack mono- and tri-chloroacetate. The organisms of groups B and C grew only feebly in ordinary media. The decomposition of monochloroacetate, trichloroacetate, and  $\alpha$ -dichloropropionate in soil was accelerated by addition of cell suspensions of groups A, B, and C, respectively. The organisms seemed to be more active in the soil than *in vitro*.

### Introduction

Several organic chloro-compounds have found widespread agricultural use as herbicides or plant growth regulators during the last decade. Most important among these are certain substituted phenoxyacetic acids, particularly 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methyl-4-chlorophenoxyacetic acid (MCPA), which are used as selective herbicides against dicotyledonous weeds. Certain simpler compounds have received attention in recent years, e.g., monochloroacetate as a ripening inducer, and trichloroacetate and  $\alpha$ -dichloropropionate as promising herbicides against 2,4-D- and MCPA-resistant weeds, such as *Agropyrum repens* and *Avena fatua*.

The biological decomposition of 2,4-D and MCPA in soil as well as *in vitro* has already been studied extensively. Several investigators (1, 2, 8, 10, 12, 14) have shown that certain bacteria are able to dissimilate these compounds, although rather little is known about the details of the processes. Jensen and Petersen (8) recovered approximately 66 to 94% of the carbon in 2,4-D and MCPA as carbon dioxide in decomposition experiments with soil, and Stapp and Spicher (14) found pure cultures of *Flavobacterium peregrium* to convert some 55% of the 2,4-D-carbon into carbon dioxide. These results indicate that the phenoxyacetic acid molecule is dissimilated *in toto* and the chlorine atoms presumably released in ionic form. Rogoff and Reid (12) recently showed in experiments with another organism that this is actually the case. Certain strains of *Micromonospora* can, according to Erikson (7), utilize *p*-dichlorobenzene as a source of carbon, and Walker (15) obtained evidence

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Contribution from the State Laboratory for Soil and Crop Research, Department of Bacteriology, Lyngby, Denmark.



that *o*-chlorophenol (but probably not *p*-chlorophenol) is decomposed biologically in the soil, although he was unable to isolate the active organisms. Walker and Wiltshire (16) later found two species of bacteria, one of them a variety of *Pseudomonas desmolytica*, able to decompose 1-chloro- and 1-bromonaphthalene, and several bacteria are known to decompose chloramphenicol (13), but no statements appear to have been made on release of chloride ions.

Much less is known about the dissimilation of simpler organic halogen compounds. The only piece of evidence seems to be an earlier extensive study by den Dooren de Jong (6), who found certain stock cultures of sporeforming and non-sporeforming bacteria and many soil bacteria, mostly pseudomonads, and some mycobacteria, able to utilize  $\alpha$ - and  $\beta$ -monobromopropionate and bromosuccinate; a smaller number of cultures also attacked  $\alpha$ -bromobutyrate and  $\alpha$ -homoisobutyrate, while none was observed to utilize trichloroacetate, trichlorobutyrate, or monobromoacetate. Growth in agar medium with 0.5% Ca salt of the various acids was used as the sole criterion of dissimilation.

The present contribution deals with the isolation and preliminary study of certain organisms able to dissimilate mono- and tri-chloroacetate and dichloropropionate, as an aspect of the fate of these compounds in the soil when used as herbicides.

### Methods

The following solution (here called "basal medium") was used in most experiments:  $(\text{NH}_4)_2\text{SO}_4$ , 0.05%;  $\text{K}_2\text{HPO}_4$ , 0.05%;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.02%;  $\text{CaSO}_4$ , 0.02%;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.005% (all w/v); in distilled water. In other cases a soil extract medium was used. It was composed of equal volumes of distilled water and soil extract (prepared by autoclaving 1 kg. of garden soil with 2 liters of water), plus  $(\text{NH}_4)_2\text{SO}_4$ , 0.05%;  $\text{K}_2\text{HPO}_4$ , 0.05%;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.02% (w/v).

The medium was usually dispensed in 5-ml. portions in test tubes as 4 ml. of basal or soil extract medium and 1 ml. solution of the chloro-organic acids as sodium salts plus various enrichments of the basal medium (amino acids, vitamins, yeast extract, etc.). The basal and soil extract media were sterilized by autoclaving, and the salts of the chloro-organic acids were added aseptically from stock solutions sterilized by filtration, to give a final concentration of 0.02 to 0.05 *M*. An excess of sterile calcium carbonate was added to media with di- and tri-chloroacetate and dichloropropionate in order to offset the acidification due to the production of hydrochloric acid on dissimilation of the acid radical. Inoculum was provided as one drop per tube of a thin suspension of cells from agar slope cultures. The temperature of incubation was 25° C. unless otherwise stated.

Chloride and bromide in liquid media were determined by the Mohr method, i.e. titration with 0.01 *N* silver nitrate and potassium chromate as an indicator. Soil extract medium was decolorized by filtration over active charcoal before the titration. The results in the tables represent averages of duplicate



cultures. Tubes with sterile medium were titrated as blanks along with the cultures; no appreciable spontaneous decomposition of the chloro-organic acids took place within 3 to 4 weeks.

Chloride in soil was determined electrometrically according to the method of Benjaminsen and Jensen (3) and expressed as p.p.m. in dry soil.

Production of carbon dioxide in soil was measured by the method of Petersen (11): 50-g. portions of moist soil in copper mesh bags were suspended in 2-liter Erlenmeyer flasks over dilute (approximately 0.05 *N*) barium hydroxide solution in which the carbon dioxide was caught and determined by titration with 0.04545 *N* hydrochloric acid.

Oxygen uptake by cell suspensions was measured by the conventional Warburg technique and calculated on the basis of cell nitrogen determined by micro-Kjeldahl analysis.

### Experiments with Monochloroacetate

#### *Isolation and General Properties of the Organisms*

Basal medium with 0.04 *M* monochloroacetate was inoculated with fresh soil and incubated; a visible turbidity developed after 1 to 2 weeks, and, after a transfer to fresh medium, platings were made on a corresponding solid medium with 2% agar. Five strains of bacteria were isolated, three (A, K, and T) from field soils, one (H) from garden soil, and one (R) from reclaimed salt marsh soil. None of these soils had previously been treated with monochloroacetate.

All five strains proved similar. Morphologically they are small rods, approximately  $2-3 \times 0.4-0.5 \mu$ , without endospores, Gram-negative, and vigorously motile, with one to four polar flagella; very short, almost coccoid rods arise in the monochloroacetate solution. The growth on nutrient agar is moderately good, smooth and glistening, whitish to very pale yellow, of a sticky consistency. A similar, somewhat thinner and more yellow, growth is produced on basal medium agar with monochloroacetate. A very abundant, whitish, and moist growth arises on nutrient agar with 1% glucose. Broth cultures show first a faint uniform turbidity, later a small viscid sediment. Stab cultures in nutrient gelatin show nailhead-like growth without liquefaction. Two strains (K and T) show a feeble acid formation from glucose and galactose in peptone water; maltose, sucrose, lactose, glycerol, and mannitol are not fermented. Starch is not hydrolyzed. Growth takes place only aerobically. Indole is not formed. One strain (A) reduces nitrate to nitrite. Optimum temperature is about 25° to 30° C.; growth at 37° C. is feeble, or lacking (strain K). A slow but vigorous growth takes place on glucose agar at 8°-10° C. but not at 1°-2° C. The strains grow on basal monochloroacetate agar within the limits of pH 5.7 to 8.0 (not at pH 5.2 and 8.5); growth on glucose agar is still vigorous at pH 5.2. Numerous carbohydrates and organic acids (as sodium salts) were tested as sources of carbon on basal medium agar; only glucose and mannitol gave abundant growth, while lactate and pyruvate, besides monochloroacetate, gave a moderate

growth of all strains; strain R also utilized citrate and benzoate. Growth with 0.02–0.1 *M* monochloroacetate is relatively good and accompanied by chloride formation in the agar (spot test with silver nitrate solution); 0.2 *M* monochloroacetate suppresses growth partially or completely. No soluble pigment is formed in any medium.

The organism is not readily identifiable but seems to be a non-fluorescent, non-proteolytic *Pseudomonas* (or *Xanthomonas* sp.). Among the species listed in Bergey's Manual (4) it seems to resemble *P. desmolytica* most closely, but a strain of this organism (received from Dr. N. Walker, Rothamsted Experimental Station, and decomposing chloronaphthalene) proved rather different, producing a definite fluorescent pigment and unable to attack monochloroacetate.

#### *Decomposition of Monochloroacetate*

The organisms are able to grow and produce chloride in the basal medium plus monochloroacetate, but the growth is slow and irregular. Further addition of 0.05% yeast extract results in rapid and vigorous growth (uniform turbidity and 89–97% ionization of the chlorine) within 3 weeks. Peptone has a similar effect (Table I). The activity varies somewhat in the different strains, of which A appears to be the most active. The effect of peptone or yeast extract can be replaced by numerous amino acids (glycine, alanine, leucine, arginine, aspartic acid, asparagine, glutamic acid, lysine, histidine, cysteine, or methionine) in a concentration of 0.02%. Vitamins (thiamine, nicotinic acid, biotin, B<sub>12</sub>) have no appreciable effect.

As shown in Table I,  $\alpha$ -monochloropropionate is also decomposed, but less readily than the acetate. The  $\beta$ -compound appeared somewhat unstable in sterile solution, and was not attacked appreciably. Monobromoacetate is decomposed at low concentration as readily as the chloro-compound by two strains, but suppresses growth at 0.05 *M*. Unlike the chloro-compound it showed some spontaneous decomposition in sterile medium (8% ionization

TABLE I  
DECOMPOSITION OF MONOCHLOROACETATE,  $\alpha$ -MONOCHLOROPROPIONATE, AND  
MONOBROMOACETATE IN BASAL MEDIUM PLUS PEPTONE BY  
VARIOUS STRAINS OF *Pseudomonas* SP.

Substrate	Incubation, days	% Cl or Bri onized by strain:				
		A	K	T	H	R
Monochloroacetate 0.05 <i>M</i> , peptone 0.08%	4	43	31	12	5	6
	8	78	83	24	13	35
	12	96	92	22	44	58
	16	100	96	84	76	65
Monochloropropionate 0.025 <i>M</i> , peptone 0.04%	3	18	—	16	—	3
	7	36	—	42	—	11
	14	47	—	48	—	42
Monobromoacetate 0.02 <i>M</i> , peptone 0.08%	4	28	—	24	—	6
	8	66	—	70	—	16
	16	96	—	90	—	10

after 16 days). Dichloroacetate and dichloropropionate were attacked only feebly with some 10 to 13% ionization of the chlorine within 3 weeks, and trichloroacetate, *o*-chlorobenzoate, and 2,4-D apparently not at all.

Respirometric experiments (Fig. 1) showed that washed cells of strain A, grown on glucose-nutrient agar (4 days, 25° C.), are able to oxidize monochloroacetate without any evidence of adaptation, although less vigorously than glucose. With cells grown on monochloroacetate agar (8 days, 25° C.) the oxidation rates of monochloroacetate and glucose are reversed, the

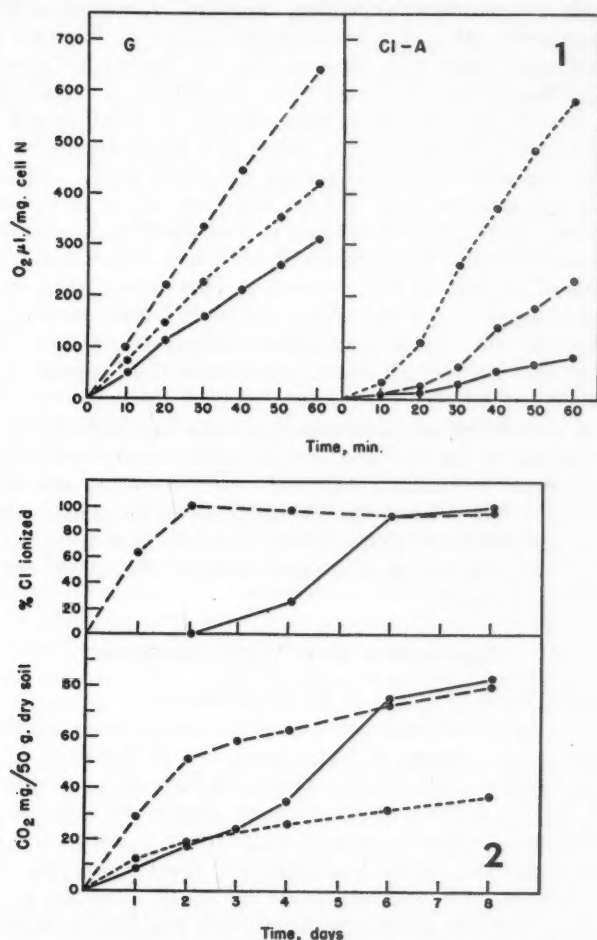


FIG. 1. Oxygen uptake by cells of *Pseudomonas* sp. (A). G, glucose-grown cells, CI-A, monochloroacetate-grown cells. —, endogenous respiration. ----, + 0.01 M glucose. ...., + 0.01 M monochloroacetate. Temp. 30° C. Phosphate buffer M/60, pH 7.4.

FIG. 2. Carbon dioxide evolution and chloride production in soil plus monochloroacetate. ...., soil without addition (control). —, + 0.05% monochloroacetic acid. ----, + 0.05% monochloroacetic acid + cell suspension of *Pseudomonas* sp. (R).

homologous substrate now being attacked very vigorously. The enzyme complex required for monochloroacetate dissimilation thus appears to be present to some extent in glucose-grown cells, but monochloroacetate medium induces a much stronger formation of the enzyme.

The organism is active in the decomposition of monochloroacetate in the soil as well as *in vitro*, as shown by an experiment reported in Fig. 2. A loamy garden soil of pH 7.2 was given an addition of 0.1% monochloroacetic acid (as sodium salt, and calculated on the basis of air-dry soil), and adjusted to approximately two-thirds water-holding capacity. A portion of this treated soil was inoculated with a thin cell suspension of strain R grown on monochloroacetate agar (other tests showed that the inoculation alone did not significantly influence the soil metabolism). Production of carbon dioxide and of ionized chloride was then determined during an 8-day period at 25° C. The addition of chloroacetate alone results in a slight depression of carbon dioxide compared with the control soil for the first 2 days: a rapid increase then sets in and subsides after 6 days, when the excess in carbon dioxide production over control soil corresponds to a release of approximately 90% of the chloroacetate-carbon. Coincident with the rise in carbon dioxide evolution, the ionization of the chlorine begins, and it reaches almost 100% after 6 days. When bacterial suspension is also added, the lag in carbon dioxide production is eliminated, and the chlorine is completely ionized after 2 days. Under the conditions of this experiment monochloroacetate thus appears to be very readily dissimilated in soil.

Ability to dissimilate monochloroacetate does not seem to be common. About 150 strains of bacteria and actinomycetes, partly collection cultures and partly random soil isolates from non-selective media, were tested with negative results for growth and chloride formation on monochloroacetate agar. However, a few strains of *Trichoderma viride*, isolated from acid soil in monochloroacetate solution, showed some evidence of activity, but have not yet been examined closely.

### Experiments with Trichloroacetate

#### *Isolation and General Properties of the Organism*

Attempts to isolate an organism capable of decomposing trichloroacetate through enrichment cultures in basal medium with 0.02–0.05 *M* trichloroacetate gave negative results (cf. den Dooren de Jong (6)). Garden soil with addition of 0.05 or 0.010% trichloroacetate showed no increase in carbon dioxide evolution or chloride content for the first 4 weeks, but the latter increased strongly after further incubation for about 2 months. Plating on basal agar medium plus trichloroacetate led to the isolation of an active bacterium that was first found associated with two strains of *Streptomyces* (these proved inactive in pure culture). Preliminary tests showed that pure cultures of the bacterium did not attack trichloroacetate in the basal medium, but did so in association with the two strains of *Streptomyces*, or if 0.10% peptone was added to the basal medium.

The active organism appears morphologically as irregular, non-sporeforming rods, in trichloroacetate medium  $0.4-0.6 \times 1.0-3.5 \mu$ , on nutrient agar up to  $3-6 \mu$ , curved and clavate, of *Corynebacterium*- or *Arthrobacter*-like type, but Gram-negative, sometimes with faintly Gram-positive granules, and with vibratory movement but no definite locomotion. Growth is very slow and scant in all media, especially just after isolation. Streak cultures on nutrient agar with or without 1% glucose show a slight, narrow, smooth, white, and pasty growth that improves somewhat on continued transfer but still remains feeble. A faint uniform turbidity arises in nutrient broth. Gelatin is not liquefied. No growth takes place in milk or on potato slices. Acid is produced from galactose but not from other sugars (in soil extract plus yeast extract and 0.5% carbohydrate). Starch is not hydrolyzed. Indole is not formed. Nitrate is not reduced to nitrite. The organism is aerobic (acid reaction appears only on the surface of deep-tube cultures in soil extract agar plus trichloroacetate and bromocresol-purple).

It does not yet seem possible to define the taxonomic position of the organism, which is here provisionally called "bacterium 3-CI".

#### *Decomposition of Trichloroacetate*

Platings from the mixed cultures of bacterium 3-CI and the streptomycetes on basal agar plus trichloroacetate and calcium carbonate showed that the growth of the bacterium started around the *Streptomyces* colonies where the calcium carbonate was dissolved and spot tests showed chloride reaction. This suggested that the streptomycetes elaborated an essential nitrilite required by the bacterium. Experiments recorded in Table II show that soil extract induces an even stronger trichloroacetate dissimilation than does the association with *Streptomyces*. It seems reasonable to assume that the active *Streptomyces* factor may be vitamin B<sub>12</sub> (cf. Burton and Lochhead (5) and Lochhead and Thexton (9)); further data included in Table II show that vitamin B<sub>12</sub> indeed replaces soil extract at much the same range of concentration as in other soil bacteria (9). Only about two-thirds of the trichloroacetate appears to have been decomposed in these experiments, but other tests with lower concentrations (0.0088-0.01 M) showed practically complete ionization after 4 to 5 weeks.

Yeast extract (Difco, 0.05%) did not activate growth and chloride formation. The growth of *Streptomyces* in the basal medium was scarcely visible, but its activating factor was apparently synthesized in the medium rather than carried over with the inoculum, since heat-killed conidia of *Streptomyces* only had a slight effect (Table III). Thirteen out of 14 other cultures of *Streptomyces* and two cultures of *Nocardia* also produced an activating factor, while two cultures of *Micromonospora* and a green *Penicillium* had no significant effect. Bio-assay with *Lactobacillus leichmannii* showed that three strains of active streptomycetes were able to produce vitamin B<sub>12</sub> in amounts of 5 to 15  $\mu\text{g.}/\text{ml.}$  in glucose-yeast extract solution which contained no vitamin B<sub>12</sub> itself. (These determinations were made by Dr. E. Hoff-Jorgensen,

TABLE II  
DECOMPOSITION OF TRICHLOROACETATE IN BASAL AND SOIL EXTRACT  
MEDIUM BY BACTERIUM 3-Cl

Medium containing trichloroacetate 0.02 M	Inoculum	Incubation, days			
		7	14	21	28
		% Cl ionized			
Basal	Bact. 3-Cl	0.6	0.8	1.1	0.7
Basal	Bact. 3-Cl + <i>Streptomyces</i> sp.	5	17	12	21
Soil extract	Bact. 3-Cl	14	29	46	60
Basal + vitamin B <sub>12</sub> , µg./ml.					
0	Bact. 3-Cl	0	0	0	0
0.01	Bact. 3-Cl	2	10	10	20
0.10	Bact. 3-Cl	2	21	24	29
1.0	Bact. 3-Cl	15	33	53	63
10.0	Bact. 3-Cl	16	36	51	70

TABLE III  
INFLUENCE OF LIVING AND HEAT-KILLED INOCULUM OF *Streptomyces* SP. ON  
DECOMPOSITION OF TRICHLOROACETATE IN BASAL MEDIUM

Inoculum	% Cl ionized after:	
	14 days	28 days
Bact. 3-Cl + <i>Streptomyces</i> , living	4	43
Bact. 3-Cl + <i>Streptomyces</i> , killed	(<1)	7
Bact. 3-Cl	—	0

Biochemical Institute, University of Copenhagen.) Two batches of soil extract, however, showed no detectable B<sub>12</sub>-content; the activating factor thus seems to be some physiological analogue of vitamin B<sub>12</sub>.

Bacterium 3-Cl does not significantly attack monochloroacetate or mono- and di-chloropropionate, but has a slight effect on dichloroacetate in soil extract (about 10% ionization in 3 weeks). Calcium carbonate-free soil extract with trichloroacetate is acidified to pH 5.0–5.2, when no further chloride formation takes place; the limiting acidity thus seems to be about pH 5. Trichloroacetate concentrations of 0.05 M or higher prevent growth. Fig. 3 shows the appearance of the growth on trichloroacetate agar, where calcium carbonate is dissolved by the released hydrochloric acid.

An experiment was carried out in garden soil plus trichloroacetate corresponding to 0.05% free acid, or 332 p.p.m. Cl. One section was inoculated with a small amount of suspension of the same soil enriched with active organisms through previous incubation with trichloroacetate, and another with a thin cell suspension of bacterium 3-Cl. Table IV shows that the slow release of chloride in the uninoculated soil is accelerated by the enriched soil, and still more by the bacterial inoculum (cf. the experiment



PLATE I



FIG. 3. Growth of bacterium 3-C1 on basal agar medium plus calcium carbonate, vitamin B<sub>12</sub>, and 0.02 *M* trichloroacetate, 27 days at 25° C.



1940-1941

1942-1943

TABLE IV

DECOMPOSITION OF TRICHLOROACETATE (0.05% FREE ACID) IN SOIL

Treatment		Incubation, days		
		14	28	38
Cl, p.p.m.	Control	31	33	28
	+ trichloroacetate	31	50	186
	+ trichloroacetate + enriched soil	43	314	319
	+ trichloroacetate + bact. 3-Cl	129	317	322
Per cent added Cl ionized	+ trichloroacetate	0	5	48
	+ trichloroacetate + enriched soil	4	85	88
	+ trichloroacetate + bact. 3-Cl	30	86	89

with monochloroacetate, Fig. 2). Surprisingly enough the increased chloride formation was accompanied by only a slight increase in carbon dioxide production, which was possibly due to interaction between soil carbonate and hydrochloric acid.

It was later found possible to isolate trichloroacetate-decomposing bacteria directly from enriched soil by plating on soil extract agar with trichloroacetate and calcium carbonate, where bacterial colonies surrounded by clear zones (cf. Fig. 3) appeared after a couple of weeks. These organisms have not yet been studied in detail.

### Experiments with Dichloropropionate

#### *Isolation and General Properties of the Organism*

Attempts to obtain enrichment cultures were made by inoculating basal medium plus 0.02 *M* dichloropropionate with various soils. Chloride was produced in the medium, but a number of cultures obtained by plating on a corresponding agar medium proved inactive. A decomposition experiment was later made with garden soil plus dichloropropionate; no increased carbon dioxide or chloride content in comparison with control soil was observed for the first 4 weeks, but chloride production became strong after further incubation for about 2 months, and an active organism (strain 2CP-0) was isolated by streaking on dichloropropionate agar with soil suspension. It was later found that dichloropropionate-decomposing bacteria could be isolated from the enriched soil by plating on soil extract agar with 0.02 *M* dichloropropionate and an excess of calcium carbonate, where several bacterial colonies surrounded by clear zones were seen after a couple of weeks (cf. experiments with trichloroacetate). Four strains (2CP-1, 2, 3, and 4) were isolated in this way.

The five strains are essentially similar: non-sporeforming rods approximately 0.4–0.7  $\mu$  wide and of varying length, from 1.6 to 5.0 or even up to 8  $\mu$ , occurring singly or in pairs, often curved and irregular, sometimes with tendency to formation of branched, Y-shaped cells reminiscent of the bacteroids of the root nodule bacteria. The Gram reaction is somewhat variable but predominantly negative. Many cells, especially the short

individuals, are vigorously motile and carry one to four flagella, mostly polar but also lateral, and often attached to a corner of the cell pole. Star-shaped clusters are sometimes seen. The microscopic picture is not unlike that of a *Rhizobium*, or *Agrobacterium radiobacter*.

All five strains produce a slow but moderately good, whitish, smooth, and slimy growth on soil extract dichloropropionate agar, but grow very feebly or not at all on nutrient agar. Nutrient broth shows either no growth or at the most a very faint turbidity. Gelatin is not liquefied. No visible growth or change is seen in milk. A slight, moist, and whitish growth arises on potatoes. No acid or gas, but on the contrary a change towards alkaline reaction, is produced in soil extract, yeast extract, and 0.5% glucose, fructose, galactose, maltose, sucrose, lactose, glycerol, or mannitol; growth appears as a uniform turbidity that seems no stronger with the various sugars than in sugar-free control medium. The best sources of carbon besides dichloropropionate appear to be lactate, pyruvate, and succinate, according to tests on soil extract agar. Starch is not hydrolyzed. Indole is not formed. Nitrate is reduced to nitrite by two strains (2CP-0 and 4). The organisms are aerobic, and grow well on dichloropropionate agar at 30° C. but not at 37° C.

Taxonomically it seems that the organisms could tentatively be placed in the genus *Agrobacterium* (cf. Bergey's Manual (4)), in view of their cell morphology and the tendency to formation of "bacterial stars", although the flagellation is chiefly polar. It is not proposed to assign a species name as yet.

#### *Decomposition of Dichloropropionate*

All five strains failed to grow on dichloropropionate in basal medium (with or without addition of vitamin B<sub>12</sub>), but decomposed this compound in soil extract medium and in basal medium enriched with yeast extract or peptone (Table V). Dichloroacetate is also attacked, eventually to about the same extent as the propionate; with inoculum taken from dichloropropionate agar there is, however, hardly any chloride formation during the 1st week, which suggests that a considerable time is required for adaptation to dichloroacetate. A few tests with monochloropropionate show that this compound is also metabolized, but no more readily than the dichloro-compound; at equimolar chlorine concentration (0.04 M) it is attacked definitely slower than 0.02 M dichloropropionate.

Mono- and tri-chloroacetate are not attacked. Soil extract medium with 0.02 M dichloropropionate but without calcium carbonate is acidified to pH about 3.8. Strain 2CP-0 continues to grow on soil extract agar with 0.10 M dichloropropionate, whereas the other strains are inhibited at 0.04-0.06 M.

Respirometric experiments with washed cells of strain 2CP-3 (Fig. 4) show that lactate-grown cells (soil extract agar plus calcium lactate, 7 days, 25° C.) have only a slight oxidizing effect on dichloropropionate, while pyruvate is more rapidly oxidized. Dichloropropionate-grown cells (10 days, 25° C.) are generally less active, and the curves show a somewhat irregular course, yet there is clear indication of increased oxygen uptake with dichloropropionate,

TABLE V

DECOMPOSITION OF DICHLOROPROPIONATE, DICHLOROACETATE, AND MONOCHLOROPROPIONATE BY *Agrobacterium* SP. IN VARIOUS MEDIA

Substrate	Strain	Medium*	Per cent Cl ionized after days:			
			7	14	21	28
Dichloropropionate 0.02 M	2CP-0	SE	14	21	29	39
	"	YE	10	24	38	52
	"	P	—	—	—	34
	2CP-1	SE	—	29	—	54
	2CP-2	SE	—	28	—	54
	2CP-3	SE	13	26	39	53
	"	P	—	—	—	60
	2CP-4	SE	—	29	—	50
Dichloroacetate 0.02 M	2CP-0	SE	(1)	11	—	41
	"	YE	0	24	33	48
	"	P	—	—	—	40
	2CP-3	SE	—	29	—	49
	"	YE	0	21	36	—
	"	P	—	—	—	60
Monochloropropionate 0.04 M	2CP-0	SE	3	7	14	—
	2CP-3	YE	0	10	17	20
Monochloropropionate 0.02 M	2CP-0	SE	—	19	—	36

\*SE, soil extract medium; YE, basal medium + 0.05% yeast extract; P, basal medium + 0.08% peptone.

which shows that the specific enzyme is an adaptive one. Pyruvate is even more readily oxidized by the dichloropropionate-grown cells; adaptation to pyruvate was to be expected, since this compound may be assumed to arise through a hydrolytic process:  $\text{CH}_3.\text{CCl}_2.\text{COOH} + \text{H}_2\text{O} \rightarrow \text{CH}_3.\text{CO}.\text{COOH} + 2\text{HCl}$ .

A decomposition experiment in soil was carried out in the same way as with mono- and tri-chloroacetate: sodium dichloropropionate was added to garden soil in an amount corresponding to 0.05% free acid on the basis of air-dry soil (253 p.p.m. Cl), and one series was "inoculated" with cell suspension of strain 2CP-0 grown for a week on dichloropropionate agar. Carbon dioxide evolution and chloride formation were followed for 24 days at 25° C. The results are seen in Fig. 5, and show that the course of decomposition is essentially the same as in the case of monochloroacetate (cf. Fig. 2), but is more protracted. The addition of cell suspension alone caused no significant increase in carbon dioxide production, and the "control" curve in Fig. 5 therefore represents the average of inoculated and uninoculated soil without dichloropropionate.

Ionization of chlorine begins in the uninoculated soil after 14 days and becomes strong after 24 days; remarkably enough the carbon dioxide production shows a slight increase only towards the very end of the experiment. The inoculation eliminates the lag period in both carbon dioxide evolution and chloride formation; the excess production of carbon dioxide over control soil almost ceases after 8 days when the excess of carbon liberated corresponds to some 60% of that added to the soil (the residual carbon may partly have

been assimilated as cell material; it is also possible that some carbon dioxide originated from interaction between soil carbonate and liberated hydrochloric acid). Corresponding to the rise in carbon dioxide evolution, about 80% of the added chlorine appears in the ionic form after 4 days, but in the later stages the ionization apparently does not exceed some 90-95%.

The percentage of ionized chlorine was calculated as excess over the initial chloride content and may be subject to some error, as the chloride content of

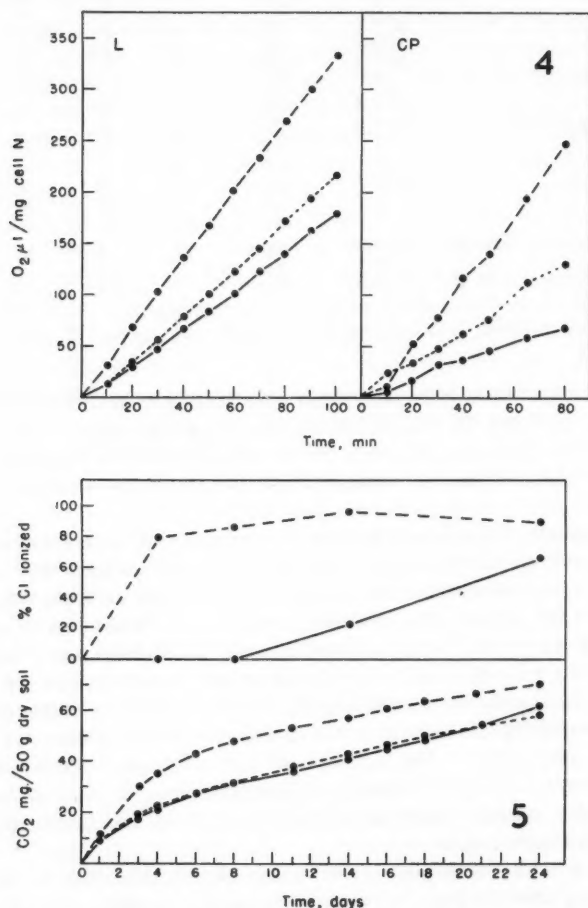


FIG. 4. Oxygen uptake by cells of *Agrobacterium*, 2CP-3. L, lactate-grown cells. CP, dichloropropionate-grown cells. —, endogenous respiration. ----, + 0.01 M sodium pyruvate. ...., + 0.01 M dichloropropionate. Temp. 30° C. Phosphate buffer M/60, pH 7.4.

FIG. 5. Carbon dioxide evolution and chloride production in soil with dichloropropionate. ...., control soil. —, + 0.05% dichloropropionic acid. ----, + 0.05% dichloropropionic acid + cell suspension of *Agrobacterium* 2CP-0.

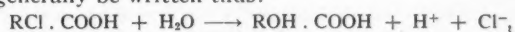
the control soil also increased somewhat during the incubation (initially 21 p.p.m., after 24 days 40 p.p.m.). The experiment was therefore repeated in a soil sample of lower chloride content, where only the chloride formation was determined. As shown in Table VI, the uninoculated soil shows no sign of chlorine ionization for 7 days, but a very steep increase occurs by the 12th day; with bacterial inoculation the ionization is quite strong even after 2 days, and practically complete after a week.

TABLE VI  
DECOMPOSITION OF DICHLOROPROPIONATE (0.05% FREE ACID) IN SOIL

Treatment		Incubation, days			
		2	4	7	12
Cl, p.p.m.	Control	9	11	9	12
	+ 2-Cl-propionate	8	10	10	262
	+ 2-Cl-propionate <i>Agrobact.</i> 2CP-0	90	250	256	—
Per cent added Cl ionized	+ 2-Cl-propionate	0	0	0	99
	+ 2-Cl-propionate <i>Agrobact.</i> 2CP-0	32	94	98	—

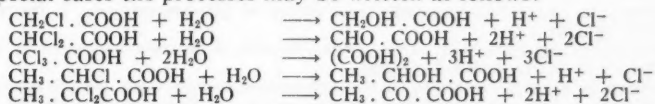
### Discussion

Although no detailed analysis of the enzymic constitution of the bacteria decomposing chloro-organic acids has yet been attempted, it seems permissible to regard their distinctive metabolic property as the ability to produce a substrate-induced enzyme catalyzing a hydrolytic process of dechlorination that may generally be written thus:



whereupon the resulting hydroxy-acid is dehydrogenated by the general respiratory mechanism and utilized as a source of energy.

In special cases the processes may be written as follows:



While the ability to use simple organic acids as sources of energy is common to many organisms, it appears that only a limited number of soil bacteria possess the ability to develop the dechlorinating enzymes. This is hardly surprising, since halogenated organic compounds are not likely to occur frequently as natural substrates for microbial decomposition in soil, although some such compounds do exist, e.g., chloramphenicol.

The rapidity with which monochloroacetate is decomposed in soil suggests that adaptation to this compound takes place readily. Decomposition of trichloroacetate and dichloropropionate is preceded by a longer period of latency, and the formation of enzymes dechlorinating these compounds thus appears to be more slowly induced. In agreement with the present results,

other experiments (according to a personal communication from Mr. H. Ingvard Petersen, Government Weed Research Department, Denmark) show that trichloroacetate and dichloropropionate, when added to the soil, retain their growth-inhibiting activity towards indicator plants for a considerably longer time than monochloroacetate.

All three groups of bacteria, and especially *Agrobacterium* sp., seem to decompose the chloro-organic acids more rapidly in the soil than *in vitro*. It may therefore be assumed, firstly, that under soil conditions permitting the growth of these organisms, the chloro-organic acids applied as herbicides will be removed biologically and not merely by leaching and/or chemical inactivation, and, secondly, that these compounds will persist for only a short time in soils that through previous application have been enriched with active organisms.

### Acknowledgments

I wish to express my thanks to Dr. E. Hoff-Jørgensen, Biochemical Institute, University of Copenhagen, for the bio-assays of vitamin B<sub>12</sub>, to Mr. J. N. Benjaminsen and Mr. J. Jensen, for help with the chloride determinations in soil, to Kemisk Værk Køge, Ltd., for a gift of pure dichloropropionic acid, and to Miss Anna-Lise Hansen for technical assistance.

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## PYOCYANINE FORMATION FROM LABELLED SUBSTRATES BY *PSEUDOMONAS AERUGINOSA*<sup>1</sup>

A. C. BLACKWOOD AND A. C. NEISH

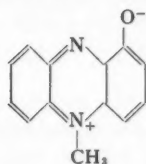
### Abstract

*Pseudomonas aeruginosa* was grown under conditions suitable for pyocyanine production in a medium containing glycerol, L-leucine, DL-alanine, calcium carbonate, salts, and small amounts of various C<sup>14</sup>-labelled substrates. A comparison of the specific activities of the cell carbon, respiratory carbon dioxide, and pyocyanine carbon showed that glycerol and dihydroxyacetone were the only substrates from which pyocyanine having a specific activity higher than the cell carbon was formed. Glucose, fructose, pyruvate, acetate, and the 13 amino acids tested were inferior in this respect. Alanine, leucine, isoleucine, and glycine were incorporated into pyocyanine more readily than the other amino acids. Phenylalanine and tyrosine, although possessing preformed rings, were poor precursors of pyocyanine and were oxidized more readily than they were assimilated. These results suggest that pyocyanine originates from trioses but gives little indication of the nature of the intermediates.

### Introduction

The production of a green color by cultures of *Pseudomonas aeruginosa* is its most widely recognized characteristic. The pigment, pyocyanine, isolated from these cultures is blue at alkaline and red at acidic pH values. Many studies have been reported (see Hays *et al.* (6) for an excellent historical review) in which this and related pigments have been investigated from different viewpoints. Extensive surveys on the effect of various constituents of the growth medium on the amount and type of pigment production have been conducted (2, 3, 7, 8) and satisfactory media have been devised. With selected strains and the correct medium high yields of this chloroform-soluble pigment are obtained. The media suggested discourage rapid production of other pigments which are known to be formed by this organism (5, 7, 8, 10).

The pigment was first isolated and characterized in 1924 by Wrede and Strack (12) and its properties described. The structural formula given below



is now generally accepted as correct. Pyocyanine has some importance as an antibiotic (11) and as a contaminant causing pigmentation of wool (4). It is easily oxidized and reduced and can take part in the hydrogen transport

<sup>1</sup>Manuscript received September 9, 1956.

Contribution from the National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan. A report of these data was presented at the Sixth Annual Meeting of the Canadian Society of Microbiologists held in Ste. Anne de Bellevue, Que., June 21-23, 1956.

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system (9). However, no studies of the mechanism of formation of this pigment have been reported and the aim of the investigation reported in this paper was to determine by use of labelled substrates the most probable precursors of pyocyanine.

## Materials and Methods

### Culture

The strain of *Pseudomonas aeruginosa* used in this study (PRL F<sub>20</sub>) was obtained from Dr. J. J. R. Campbell, Dairy Department, University of British Columbia, labelled ATCC 9027.

### C<sup>14</sup>-labelled Substrates

All of these labelled compounds were obtained from Atomic Energy of Canada, Ltd., except dihydroxyacetone-1,3-C<sup>14</sup>, which was prepared from glycerol-1,3-C<sup>14</sup> by oxidation with *Acetobacter suboxydans* PRL G<sub>1</sub>.

### Medium

The medium used was that of Burton *et al.* (2, 3) as modified by Hellinger (7). It contained 0.4% DL-alanine, 0.8% L-leucine, 0.2% MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.05% K<sub>2</sub>HPO<sub>4</sub>, 0.001% FeSO<sub>4</sub>·7H<sub>2</sub>O, 1.0% glycerol, and a measured amount (*ca.* 0.1%) of CaCO<sub>3</sub>. The salts, glycerol, and amino acids were sterilized separately and combined at the time of inoculation. If radioactive substrates were added, they were sterilized separately and added as desired. Bacterial growth was very rapid and maximum pyocyanine formation occurred after 48 to 72 hours' fermentation time. No detectable amount of fluorescein was found, but on longer incubation a dark red, water-soluble, chloroform-insoluble pigment was produced in large amounts.

### Fermentation Conditions

The medium was dispensed in 100 ml. amounts in a 500 ml. Erlenmeyer and was incubated at 30° C. on a rotary shaker at 200 r.p.m. Carbon dioxide was collected by passing a stream of sterile carbon dioxide-free air through the fermentation flask at 100 ml. per minute and then through a companion flask (500 ml. Erlenmeyer) containing 50 ml. of 1 N sodium hydroxide.

### Isolation of Products

The fermentation medium was acidified with 1.5 ml. of 5 N hydrochloric acid and aeration continued for 1 hour. The alkali containing the carbon dioxide was made to a definite volume and an aliquot analyzed for total carbonate and C<sup>14</sup> (1). A correction was applied to the respiratory carbon dioxide for the calcium carbonate added to the medium. The acidified fermentation solution was centrifuged and the cells were washed twice with water and analyzed by combustion with Van Slyke fluid for total carbon and C<sup>14</sup> (1). The supernatant solution was neutralized with solid sodium bicarbonate and the pyocyanine extracted in a separatory funnel with three 40 ml. portions of chloroform (12). The combined extracts were extracted once with water containing enough hydrochloric acid to convert all the

pyocyanine to the water-soluble salt. This red aqueous solution was neutralized with solid sodium bicarbonate and again extracted with chloroform as before. The chloroform extracts were combined, dried with anhydrous sodium sulphate, and evaporated to give the crude pyocyanine as a dark blue solid.

The crude pyocyanine (about 20 mg.) was dissolved in a small amount of chloroform and filtered through a  $1.1 \times 11$  cm. column of alumina (Merck's chromatographic grade). The pyocyanine was absorbed as a deep blue band which moved slowly down the column as it was washed with chloroform. The column was washed with three volumes of chloroform and the pyocyanine eluted as a single sharp band with chloroform-ethanol mixture (9 : 1, v/v). The effluent containing the pyocyanine was evaporated to give purified pyocyanine. This was then combusted for measurement of  $C^{14}$  (1). The purified pyocyanine represented about two-thirds the weight of the crude pyocyanine fraction. There was no indication of other pigments and the other third presumably consists of colorless compounds eliminated by the chromatographic purification.

#### *Conversion of Pyocyanine to 1-Hydroxyphenazine*

A sample of purified pyocyanine, obtained from cultures containing glycerol-1,3- $C^{14}$ , was degraded to 1-hydroxyphenazine by alkali and hydrogen peroxide essentially as described by Wrede and Strack (13). The pyocyanine (47 mg.; 0.228  $\mu\text{c./mM.}$  of carbon) was dissolved in 15 ml. of water and 0.5 ml. of 50% (w/v) sodium hydroxide and four drops of 30% hydrogen peroxide were added. After 1 hour another three drops of peroxide were added and after another hour 0.5 ml. of 85% phosphoric acid. The precipitate was filtered, resuspended in water containing a little phosphoric acid, and extracted three times with ether in a separatory funnel. The ether extract was dried by anhydrous sodium sulphate and evaporated to give 30 mg. of crude 1-hydroxyphenazine (m.p. 142–152° C.). A portion (13 mg.) of this crude material was sublimed at 110–130° C. at 0.01 mm. pressure, to give 10 mg. of purified 1-hydroxyphenazine (m.p. 155–156° C.). Combustion of this gave carbon dioxide with 0.225  $\mu\text{c./mM.}$  This specific activity is practically the same as that of the carbon dioxide obtained by combustion of the pyocyanine (0.228  $\mu\text{c./mM.}$ ).

The methyl carbon lost on this degradation was converted to formic acid (13). The filtrate from the precipitation of crude 1-hydroxyphenazine was diluted with 50 ml. of water and refluxed for 90 minutes with 4 ml. of 85% phosphoric acid and 3 g. of mercuric oxide. This gave 0.202 mM. (90% recovery) of carbon dioxide with an activity of 0.149  $\mu\text{c./mM.}$  This is an estimate of the activity of the formic acid and thus of the methyl carbon of pyocyanine.

#### **Results and Discussion**

Pyocyanine was formed in good yields only when the organism was grown on a complex medium containing, in addition to salts, three different carbon compounds. Trials with resting cells, grown on the complete medium, failed

to produce enough pyocyanine from any single carbon source; it was necessary to add salts and the three carbon sources, glycerol, alanine, and leucine. The labelled substrates tested thus supplied only a small fraction of the total carbon and entered the metabolic pathways in competition with the regular constituents of the medium.

An idea of the metabolic fate of these labelled substrates can be obtained by comparing the relative specific activities given in Table I of the cell carbon, the respiratory carbon dioxide, and the pyocyanine carbon. These data indicate to what extent the labelled substrates were assimilated into one or more of the numerous cell constituents, were respired to carbon dioxide, and were used for the formation of pyocyanine.

Glycerol and dihydroxyacetone were the only substrates which formed pyocyanine with a specific activity higher than the average level of the cell constituents. These compounds were much more easily converted to pyocyanine than fructose, glucose, pyruvate, or acetate. The pyocyanine

TABLE I  
UTILIZATION OF LABELLED SUBSTRATES BY *Pseudomonas aeruginosa*  
DURING PYOCYANINE PRODUCTION

Labelled substrate*	Total $\mu$ c. added†	Specific activity of cell carbon ( $\mu$ c./mM.)‡	Relative specific activity (cell carbon = 1.00)	
			Pyocyanine carbon	Respiratory carbon
Glycerol-1,3-C <sup>14</sup>	9.80	0.120	1.91	0.83
Dihydroxyacetone-1,3-C <sup>14</sup>	3.41	0.014	1.73	1.09
L-Alanine	4.33	0.057	0.35	1.49
L-Isoleucine§	4.10	0.048	0.33	1.23
L-Leucine	3.98	0.039	0.25	1.05
D-Fructose	4.9	0.066	0.22	1.01
L-Glycine	4.56	0.096	0.17	0.23
Sodium pyruvate-2-C <sup>14</sup>	5.0	0.052	0.09	1.28
D-Glucose	4.88	0.035	0.06	1.37
Sodium acetate-1-C <sup>14</sup>	10.05	0.065	0.01	2.85
Sodium acetate-2-C <sup>14</sup>	8.75	0.078	0.05	1.21
L-Tyrosine	3.78	0.051	0.05	2.05
L-Glutamic acid	4.90	0.040	0.04	2.20
L-Aspartic acid	4.85	0.038	0.05	2.28
L-Arginine	4.21	0.037	0.04	1.94
L-Proline	4.37	0.056	0.06	1.21
L-Phenylalanine	6.75	0.066	0.03	1.31
L-Lysine	4.72	0.050	0.03	0.72
L-Threonine	5.18	0.077	0.03	0.28
L-Valine§	3.91	0.068	0.03	0.22

\*Uniformly labelled with C<sup>14</sup> unless otherwise indicated.

†0.050 millimole of labelled substrate was added per 100 ml. of medium.

‡In a typical fermentation about 23 mM. of CO<sub>2</sub>, 12 mM. of cell carbon, and 1 mM. of pyocyanine carbon were obtained per 100 ml. of medium.

§The DL acid was used as the carrier, thus each fermentation had 0.049 mM. of unlabelled D acid and 0.050 mM. of labelled L acid.

formed from glycerol was labelled mainly in the phenazine nucleus as shown by the degradation to 1-hydroxyphenazine described above. It thus appears that pyocyanine can be formed from glycerol more readily than from the major intermediates of glucose metabolism. This suggests that non-phosphorylated trioses (e.g. dihydroxyacetone) may be converted to six carbon compounds which could condense to form the phenazine ring. However, a discussion of the chemical nature of possible intermediates would be premature at this time.

Although none of the amino acids were utilized as readily for pyocyanine synthesis as glycerol there were distinct differences between them. Alanine, leucine, isoleucine, and glycine were used more readily than the others for pyocyanine production. Phenylalanine and tyrosine, although possessing preformed rings, were among the least efficient pyocyanine precursors of all the compounds tested. However, they were readily oxidized to carbon dioxide as were arginine, glutamic acid, and aspartic acid.

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## A STUDY OF THE DISTRIBUTION AND THE EFFECTS OF BACTERIOPHAGE OF ROOT NODULE BACTERIA IN THE SOIL<sup>1</sup>

JANINA KLECZKOWSKA

### Abstract

Bacteriophage for clover nodule bacteria can be found on roots and nodules of all naturally grown clover plants and also in the soil surrounding the roots, but not in soil without clover plants. Alternative hosts for the phage of clover bacteria are pea bacteria, and vice versa. The bacteria and the phage are heterogeneous in the sense that only a proportion of strains of clover bacteria and of pea bacteria are susceptible to lysis by a given race of phage, and only a proportion of races of phage can lyse a given bacterial strain. There does not seem to be any association between the susceptibility of bacterial strains to lysis by phage and any other features such as antigenic structure and effectiveness in nitrogen fixation. There may be an association with avirulence, i.e. inability to infect the host plant. The behavior of phage-bacterial mixtures depends on the surrounding medium. The longevity of phage in soil or in a soil-like medium such as a vermiculite mixture is relatively short, and the effect of phage can be localized so that phage-susceptible bacteria and the phage can exist close to each other without any apparent interaction. However, as long as the phage is present, phage-resistant bacterial mutants are usually present also. The phage-resistant mutants may also be mutants in other respects such as effectiveness in nitrogen fixation. In the presence of weakened phage, bacterial mutants were found to occur that differ from the parent form in effectiveness but resemble it in susceptibility to the phage.

### Introduction

Demolon and Dunez (1) found that bacteriophage of lucerne nodule bacteria is present in root nodules, roots, and stems of all old lucerne plants, and also in the soil surrounding the roots, but not at distances longer than about 30 cm. They also claimed that bacteriophage destroys the bacteria in the root nodules and in the surrounding soil, and thus interferes with nodulation, with nitrogen fixation, and with normal development of the plants in nitrogen-deficient soils or other media. The soil condition known as "lucerne sickness of soil" (*la fatigue des luzernières*) is, according to Demolon and Dunez, caused by the destruction of lucerne nodule bacteria by bacteriophage.

It is not known whether the results of Demolon and Dunez apply to clover nodule bacteria and clover plants. That some of them do not seem to apply is shown by the fact that Grijns (2) and Laird (7) found that bacteriophage of clover bacteria had no effect on nodulation, nitrogen fixation, and normal growth of clover plants. This paper describes a study of the distribution of bacteriophage of clover nodule bacteria in the soil and of the role it may play there.

### Methods

The method of isolating phages from soil and from clover roots and nodules was similar to that described by Vandecaveye and Katznelson (9). A clover plant was dug out together with a lump of soil. The soil was separated from

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the roots and sifted. The roots were cleaned of the remainder of the soil by washing in water. Twelve nodules were taken for isolation of bacteria and the remaining nodules together with the roots were ground in a mortar. Five milliliters of tap water were added to each gram of the sifted soil and to each gram of the ground roots and nodules. The mixtures were poured into flasks and shaken vigorously by hand for 5 minutes. They were then filtered through filter paper and the filtrates filtered again through Chamberland L3 porcelain filters. One milliliter of the final filtrate was added to each of a series of tubes each containing 10 ml. of a 24 hour culture of a bacterial test strain in the liquid medium. The tubes were incubated at 28° C. for 3 days and the results read at intervals of 24 hours. The absence of bacterial lysis after this time was taken as evidence of the absence of phage virulent for the particular bacterial test strain. Sixteen different bacterial test strains were usually used: four classical laboratory strains and 12 bacterial cultures freshly isolated from the nodules of the plants tested. When soils in which no clover plants had grown were tested, 16 bacterial test strains were also used: four classical laboratory strains and 12 bacterial cultures isolated from nodules of the nearest clover plants that grew in the neighborhood.

If any of the bacterial test strains were lysed by the filtrate of a soil extract, a quantitative assay of phage in the extract was made by the previously described plaque count method (3), using one of the susceptible strains.

The methods of isolation of bacterial strains from root nodules and of testing bacterial strains for effectiveness of nitrogen fixation in root nodules, and the liquid and agar media, were the same as those used previously (4).

### Results

#### *Occurrence and Distribution of Phage in the Soil*

Phage was detected in all 10 extracts from clover roots and nodules and in all 10 extracts from samples of soil in which the clover plants had grown. No phage was detected in any of six extracts tested from soil in which no clover plants (or any other leguminous plants) had grown. Half the number of samples of each kind were taken from fields and the other half from pots kept in the greenhouse.

The phages that were present in the extracts tested did not lyse all the bacterial test strains. Out of the four classical laboratory strains, usually not more than three were lysed, some more frequently than others, and out of 12 bacterial cultures freshly isolated from root nodules for each particular case, usually not more than eight were lysed. Phage concentrations usually corresponded to 1-2 plaques per ml. of soil extract. The phage isolated from the experimental plot at Rothamsted, where clover plants had continuously grown for 100 years, had an exceptionally wide host range, lysing all the four classical bacterial strains and 10 out of 12 freshly isolated bacterial cultures. Phage concentration was also exceptionally high, corresponding to 20 plaques per ml. of soil extract.

The 100 year clover field at Rothamsted showed no signs of "clover sickness", the plants being quite normal in appearance and growth. Of all

the other tested soils in which clover plants had grown, only those in four pots were "clover sick". The phages of these soils did not differ consistently from those of the other soils either in host range or concentration. Thus no evidence was obtained that "clover sickness" of soil is due to bacteriophage.

The implications of the above results may be interpreted after taking into account two facts: the loss of a proportion of phage during passage through a porcelain filter, and the heterogeneity of clover nodule bacteria and of their phages, which means that only a proportion of strains of the bacteria are lysed by a given "race" of phage and only a proportion of "races" of phage for clover nodule bacteria can lyse a given bacterial strain. To estimate the loss of phage during filtration, a phage stock culture was diluted in water and filtered through a Chamberland L3 filter. Phage was assayed in the fluid before and after filtration. The proportion of phage lost during filtration depended on phage concentration. To obtain a filtrate with any phage concentration corresponding to between 1 and 10 plaques per ml. (as in filtered soil extracts), about 1.5 times more concentrated fluids had to be filtered. Thus about 30% of phage was lost by filtration. To minimize the uncertainty of results due to heterogeneity of phages and bacterial strains, 16 different bacterial strains were used for detecting the presence of phage in the tested samples. As 12 of the strains were isolated from the same material or from material collected nearby, it seemed likely that at least some of these strains would be susceptible to the lytic effect of phage that may be present in the sample tested. The fact that phage was detected in all the soils where clover plants had grown and in none of those without clover plants can be taken as evidence that phage for clover nodule bacteria usually occurs only in soils where clover plants grow.

Assays of phage for clover nodule bacteria in the soil give only minimum values. Assuming that every particle of phage that can lyse the bacterial strain used for plating forms a plaque, the assays will still give low values because of the loss of phage during filtration and because of heterogeneity of phages and bacterial strains. The loss during filtration can be allowed for by multiplying the resulting figures by a factor (in this case by 1.5), but no such factor can be found to allow for heterogeneity. If a soil sample contains a mixture of different "races" of phages for clover nodule bacteria, the phage particles of only those "races" that can lyse the bacterial strain used for plating will form plaques. The proportions of numbers of particles of such phages to the total numbers of particles of all phages for clover nodule bacteria in different soil samples are unknown. The figures that are obtained from the assays can, therefore, apply only to concentrations of phage that can lyse the bacterial strain used for plating.

#### *Phage Susceptibility Relationships Among Nodule Bacteria of Different Host Inoculation Groups*

The problem of distribution of phages for clover nodule bacteria in the soil is connected with the problem of alternative hosts. Table I shows that pea nodule bacteria are alternative hosts. Two different phages lysed widely

TABLE I  
SUSCEPTIBILITY TO LYSIS BY TWO PHAGES OF BACTERIAL STRAINS OF  
DIFFERENT INOCULATION GROUPS

	Inoculation groups of bacteria tested				
	Clover	Pea	Lucerne	Lupine	Soybean
Total number of strains tested	30	16	5	5	4
Number of strains lysed by phage:					
I	26	12	0	0	0
II	3	2	0	0	0

NOTE: Phage I was isolated with a strain of clover bacteria, and phage II with a strain of pea bacteria. All the strains that were lysed by phage II were also lysed by phage I.

different proportions of tested bacterial strains, but each lysed about an equal proportion of strains of each of the two inoculation groups, although one of the phages (I) was originally isolated as a phage for clover nodule bacteria and the other (II) for pea nodule bacteria. As each phage was virulent against about equal proportions of strains of both inoculation groups of bacteria, there does not seem to be any reason to distinguish between phages for clover bacteria and for pea bacteria. The phages did not lyse any of the tested strains of nodule bacteria of three other inoculation groups: lucerne, lupine, and soybean. However, as the numbers of these strains were small, generalizations would not be safe.

The similarity in phage susceptibility between strains of clover and pea inoculation groups of bacteria is paralleled by serological relationships. Kleczkowski and Thornton (6) found that clover and pea bacteria are not serologically distinguishable. Both groups are serologically heterogeneous, containing groups of strains serologically related among themselves but unrelated to strains of other groups. The groups of serologically related strains contain strains of both clover and pea bacteria. However, no relationship between antigenic structure and susceptibility to lysis by a phage was found. Marshall and Vincent (8) on the other hand, working with other strains of clover bacteria and other "races" of phage, did find such a relationship. It is possible that different structural elements that condition susceptibility of bacterial cells to different phages may or may not be associated with antigenic structures. Further evidence of the close relationship between clover and pea bacteria is the fact that some of the strains can cross-infect (10, 5).

#### *Susceptibility to Lysis by Phages and Other Characteristics of Bacterial Strains*

Table II was compiled to determine whether there is any association between susceptibility to lysis by a phage and other characteristics of bacterial strains, such as antigenic structure, ability to infect the host plant (virulence), and effectiveness in nitrogen fixation in root nodules. The antiserum used for the agglutination tests was prepared against a bacterial strain that was highly effective in nitrogen fixation and susceptible to lysis by the phage used

TABLE II  
DISTRIBUTION OF VARIOUS CHARACTERISTICS AMONG 17 STRAINS OF  
CLOVER NODULE BACTERIA

Lysis by phage	Agglutination by antiserum	Nitrogen fixation		Avirulent
		Effective	Ineffective	
+	+	3	1	0
+	-	3	2	0
-	+	1	0	1
-	-	2	1	3

NOTE: The figures show numbers of strains that fall into groups of different combinations of features.

in the tests. There were susceptible and resistant strains as well as effective and ineffective ones among those that were and those that were not agglutinated by the antiserum, and also among those that were and those that were not lysed by the phage. It can be concluded, therefore, that neither susceptibility to lysis by phage, nor effectiveness in nitrogen fixation, is associated with any particular antigenic structure, and that there is no association between susceptibility to phage and effectiveness or ineffectiveness in nitrogen fixation. Virulence, i.e. the ability of bacterial strains to infect the host plant (irrespective of effectiveness or ineffectiveness in nitrogen fixation), was shown by strains that were and were not lysed by the phage, and by strains that were and were not agglutinated by the antiserum. Virulence is not, therefore, associated with any particular antigenic structure, or with susceptibility or resistance to phage. Avirulent bacterial strains, on the other hand, occurred among those that were resistant to lysis by phage. As there were four avirulent strains, the probability of this occurring by chance was  $1/16$ . It is possible, therefore, that avirulence might be associated with resistance to lysis by phage, although the reverse is obviously not true. This could be further tested because there were at Rothamsted a number of virulent and avirulent successive derivatives of one bacterial strain. Out of six avirulent derivatives only one was lysed by the phage, and out of six virulent derivatives only one was not lysed. Thus the loss of virulence may be associated with the loss of susceptibility to lysis by phage.

*Effect of Environmental Conditions and the Behavior of Phage-Host Mixtures*

As phages and susceptible bacteria are found in the same samples of soil and in clover plants growing in it, conditions in the soil seem to be such that phages and bacteria can exist side by side without affecting each other. To see how far external conditions can affect phage-host interactions, comparisons were made between the behavior of phage-host mixtures in nutrient liquid or agar medium, in sterilized soil, and in a vermiculite mixture. The soil was "garden loam" with pH 6.2. The vermiculite mixture consisted of two parts of vermiculite, one part of sand, and one part of crushed flint. Both were

TABLE III  
EFFECTS OF EXTERNAL CONDITIONS ON GROWTH OF PHAGE-BACTERIAL MIXTURES AND ON APPEARANCE OF PHAGE-RESISTANT MUTANTS

Time of incubation	Medium	Bacterial strain "A12111" (effective in nitrogen fixation)			Bacterial strain "Coryn" (ineffective in nitrogen fixation)		
		Conc.* of:			Conc.* of:		
		Phage	Bact.	Resist.	Phage	Bact.	Resist.
1 month	Soil	0	$7 \times 10^4$	0	0	$3 \times 10^3$	100
	Vermiculite mixture	$3 \times 10^4$	$2 \times 10^5$	64	$3 \times 10^3$	$2 \times 10^5$	13
3 months	Liquid medium	$2 \times 10^5$	$9 \times 10^5$	100	$8 \times 10^5$	$2 \times 10^7$	100
	Soil	0	3	—	0	0	—
	Vermiculite mixture	10	200	100	$2 \times 10^4$	500	55
	Liquid medium	$5 \times 10^5$	$3 \times 10^7$	100	$5 \times 10^7$	$3 \times 10^7$	100

\*The concentrations are given in terms of numbers of phage particles (plaque count) and of viable bacterial cells (colony count) per ml. of the liquid medium or per g. of each of the other media. The initial concentrations were about  $5 \times 10^4$  for the bacterial strains and about  $5 \times 10^5$  for the phages.

†Based on the results of testing 30–40 cultures isolated from single colonies. The same cultures were also tested on plants for effectiveness in nitrogen fixation. The results of these tests are given in Table IV.

TABLE IV  
EFFECT OF EXTERNAL CONDITIONS ON APPEARANCE OF MUTANTS IN EFFECTIVENESS IN NITROGEN FIXATION IN PHAGE-BACTERIAL MIXTURES

Treatment of phage-bacterial mixtures	Bacterial strain "A12111", originally effective			Bacterial strain "Coryn", originally ineffective		
	Effectiveness in nitrogen fixation:			Effectiveness in nitrogen fixation:		
	Effect.	Intermed.	Ineffect.	Effect.	Intermed.	Ineffect.
3 months' incubation in the liquid medium	0	0	40(r)	0	0	40(r)
3 months' incubation in the vermiculite mixture	35(s)	0	1(s)	9(s)	2(s)	29(rs)
Surface inoculation of agar plates	17	0	33	0	6	47

NOTE: The figures are the numbers of isolated bacterial cultures that fell under the three headings. The same cultures were tested for susceptibility to the phages: (r) all cultures were phage resistant; (s) all cultures were phage susceptible; (rs) 22 cultures were phage resistant and 7 phage susceptible.



sterilized by autoclaving twice for 1 hour at 15 lb. pressure. Two bacterial strains were used, one of which ("A12111") was effective and the other ("Coryn") ineffective in nitrogen fixation, and two corresponding virulent phages.

Twenty-five milliliters of a water suspension of bacteria grown on agar slopes (ca.  $6 \times 10^8$  bacteria per ml.) and 2.5 ml. of a phage stock (ca.  $6 \times 10^8$  plaques per ml.) were added to 250 ml. of a sterile soil extract. The mixture was kept for  $\frac{1}{2}$  hour at room temperature to allow for combination between phage and bacteria, and then 1 ml. of the mixture was added to 10 ml. of the liquid medium or to 10 g. of soil or vermiculite mixture, whose water content was then increased by adding another 1 ml. of the soil extract. All the media were incubated at room temperature in tubes plugged with cotton wool. Determinations were made after 1 month and after 3 months of incubation; the results are given in Tables III and IV. The liquid medium was tested directly, whereas water was added to the other media (soil and vermiculite mixture) in the amount of 1 ml. for each gram, and the unclarified water extracts thus obtained were used directly. Table III shows that in the soil the phage disappeared completely during the 1st month. Bacteria that escaped the effect of the phage initially present probably multiplied for some time within the 1st month of incubation and were all susceptible to lysis by the phage. However, after the initial period of multiplication, the bacteria died out in the sterilized soil and only very few were detected after 3 months of incubation.

In the vermiculite mixture the two phage-bacterial systems behaved somewhat differently, although the general trend was similar. In one system the concentration of phage after 1 month was about 1/10th of the original, and almost no phage was detected after 3 months. The concentration of viable bacteria increased during the 1st month and about 40% of the bacteria were phage-resistant mutants. After 3 months most bacteria had died out, but those that survived were all phage susceptible. In the other system phage concentration after 1 month was 10 times the original, and after 3 months just under 1/10th of the original. The concentration of viable bacteria increased during the 1st month, about 10% being phage-resistant mutants; after 3 months most of the bacteria had died out, about half of those that survived being phage resistant. Thus, in the vermiculite mixtures bacteria susceptible to lysis by a phage can exist side by side with the phage. When the phage is at a sizable concentration, phage-resistant bacterial mutants are usually present. If phage dies out, the phage-resistant mutants seem to revert to susceptibility or die out sooner than phage-susceptible bacteria.

The liquid medium contained an abundant population of bacteria that appeared as secondary growth after the original bacterial population was lysed. All the bacteria were phage-resistant mutants and their numbers increased between 1 and 3 months of incubation. The concentration of active phage decreased during this time, but did not fall to the low level to which it usually falls in similar conditions in filtered cultures free of bacteria (i.e. to

about  $10^3$ ). This may be due to the fact that the population of phage-resistant bacteria occasionally produced phage-susceptible cells, or because accumulating bacterial metabolic products protected phage from inactivation.

Scarcity of nutrients for bacteria in the soil and in the vermiculite mixture could have been responsible for the results obtained. An experiment was set up with the soil and vermiculite mixture exactly as described above, except that a liquid nutrient medium was substituted for the soil extract. In the presence of the nutrients the bacteria all became resistant as they did in liquid media, suggesting that in the earlier experiment the persistence of susceptible bacteria was due to lack of nutrients. But the concentration of phage fell between 1 and 3 months of incubation to the level to which it usually falls in filtered liquid cultures free of bacteria. This could be explained by assuming either that bacterial metabolic products that may protect phage from inactivation in the liquid medium were adsorbed by soil particles, or that phage was adsorbed.

In addition to the tests shown in Table III, the bacterial cultures isolated after 3 months of incubation with phages in different media were also tested on plants for effectiveness in nitrogen fixation. The results are shown in Table IV, which also includes results of an experiment in which phage-bacterial mixtures were inoculated on the surface of agar plates, as described previously (4). There is evidence that phage-resistant mutants arise independently of any action of the phage, which merely reveals their presence by destroying susceptible bacteria. There is also evidence that when a bacterium is in a state of disequilibrium resulting in a mutation in one feature, a mutation in another feature is also likely to occur, although the mutations may be independent of each other (4). There may also be a reversal of one mutation without a reversal of another. Thus phage-resistant mutants may become susceptible again, but an accompanying mutation, for example, in effectiveness in nitrogen fixation, may not be reversed. Thus the bacterial mutations in the phage-bacterial mixtures, shown in Table IV, can not be considered to be caused by phage, but may merely be selected by it. It should be borne in mind, however, that the rates of mutation of the two bacterial strains ("A12111" and "Coryn") must be extremely low, so that in the absence of a selective factor such as phage, they appear perfectly stable.

In the liquid medium all the bacteria that were tested appeared uniform, all being phage resistant and all ineffective in nitrogen fixation, although one of the original strains was effective. The other two media, on the other hand, appeared to contain bacteria that differed in effectiveness in nitrogen fixation and in phage susceptibility (see also Table III).

The uniformity of the bacterial populations in the liquid medium as compared with agar can be explained by the ease of diffusion. The newly formed mutant cells are at first very slow growing, although eventually they grow as fast as their parent forms (4). In a liquid medium all bacteria except those that are phage-resistant mutants are lysed. Now, one of the slow-developing mutants may multiply faster than the others, and so its progeny

may eventually suppress multiplication of the other mutant bacteria and dominate the whole of the medium. Thus all the culture may be uniform, and all the bacteria will be like or unlike the parent strain in effectiveness of nitrogen fixation, depending on whether or not the successful mutant to phage resistance happened to be also a mutant in effectiveness in nitrogen fixation. On agar plates, on the other hand, the progenies of all the mutant bacteria are localized as colonies and so do not interfere with each other's multiplication. In the vermiculite mixture, whose water content was just sufficient to make it moist, progenies of different bacteria presumably were sufficiently localized so as not to interfere with each other as much as they do in a liquid, and so bacteria with different features can easily be found. The same reasoning applies to the soil. The presence of phage-susceptible bacteria in the same vermiculite mixtures or in the same soil cultures which also contain the phage can also be explained by assuming localization of the phage and adsorption on soil or vermiculite particles.

Mutants in effectiveness in nitrogen fixation which were found in the vermiculite mixtures were all phage susceptible. This can perhaps be explained by assuming that they were descendants of bacteria that mutated to phage resistance and also in effectiveness in nitrogen fixation, but subsequently reverted to phage susceptibility while retaining the other acquired feature.

### Discussion

The distribution studies described above show that the presence of phage in soil is closely related to that of the plant that harbors the host bacteria, but that its presence does not result in the elimination of its bacterial host.

The experiments with the vermiculite mixture showed that phage and bacteria added to such a mixture could coexist provided that no nutrients are added. The bacteria produced some resistant mutants but susceptible cells were not eliminated. The phage destroyed all but the resistant mutants in the soil when nutrients were added. The persistence of susceptible forms where no nutrient was supplied would seem therefore to be due to starvation, reducing multiplication of the bacteria and hence of the phage.

Previous work by Kleczkowska (4) has shown that some of the resistant mutants of *Rhizobium* appearing in the presence of phage differ also from the parent form in the effectiveness of the nodules that they produce. Similar changes in effectiveness may occur amongst phage-susceptible bacteria in the absence of phage, but such mutants are much more abundant amongst phage-resistant strains.

The behavior of the "Coryn" strains added with phage to the vermiculite mixture was thus at variance with past experience in that the strain produced a considerable proportion of effective or partly effective mutants (all of which were phage susceptible) but no effective mutants that were phage resistant.

With an effective parent strain, a large proportion of the mutants developed in the presence of phage have been found to be ineffective, whereas with an ineffective parent strain, relatively few effective resistant mutants appear

after treatment with phage. The presence of phage in soil carrying clover should thus result in the accumulation of ineffective, resistant mutants and hence in a correlation of resistance and ineffectivity in the wild population. But if susceptible as well as resistant mutants to effectiveness tend to appear in the presence of reduced phage activity no such correlation would be expected and in fact none has been found. The appearance of mutants influencing nodule behavior in the presence of phage may bear on the occurrence of ineffective strains in field soils, but apart from this, the present work supports the conclusion of Grijns (2) and Laird (7) that phage is not likely to have an immediately harmful effect on a clover crop.

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# HOST SPECIFICITIES OF FOUR LOTUS RHIZOBIOPHAGES<sup>1</sup>

C. W. BRUCH AND O. N. ALLEN

## Abstract

A study was made of the host affinities of four *Lotus* rhizobiophages for 91 rhizobial strains from 10 *Lotus* spp., 376 strains of other rhizobia, and 40 strains of species in the genera *Agrobacterium* and *Chromobacterium*. No one phage lysed every rhizobial strain of the *Lotus* spp.; however, all of the phages lysed every strain from *Lotus angustissimus*. None of the strains from *Lotus uliginosus* was susceptible. Among rhizobia from the other eight *Lotus* spp. all strains were lysed by at least one of the four phages. Of the 376 strains of other rhizobia, 28 strains of six plant genera were lysed by one or more of the four *Lotus* phages. None of the phages lysed any of the strains of species in the genera *Agrobacterium* and *Chromobacterium*.

## Introduction

Within the last two decades numerous reports have emphasized the use of bacteriophages in the identification and typing of bacterial strains and species. As early as 1925 Coons and Kotila (6) mentioned the applicability of this technique in studying certain plant pathogenic bacteria. Thomas (27) identified bacterial isolations from pear, apple, mountain ash, cotoneaster, and hawthorn by using a lytic agent specific for *Erwinia amylovora* (Burrill) Winslow *et al.* Quick detection of strains of *Aplanobacter stewartii* McCulloch (*Bacterium stewartii* Erw. Smith) was also afforded by strain-specific phagic agents. In later studies Thomas (28, 29) used the phage reaction to identify human and phytopathogenic bacteria. Relatedly, other reports have shown the value of phages as useful tools in the rapid typing, identification, and classification of hemolytic streptococci (9, 10, 11), *Salmonella* (23), diphtheria (21), typhoid (7), dysentery (24), staphylococci (12, 13, 14), and acid-fast microorganisms (15).

In recent years other investigators have focused attention on the application of lytic techniques in the identification of phytopathogenic bacteria. In 1948 Thornberry *et al.* (31, 32) differentiated between diverse cultures of *Xanthomonas* Dowson and related organisms by means of a phage host-specific for the former. *Xanthomonas lactucae-scariolae* (Thornberry and Anderson), distinguishable from *X. pruni* only by pathogenicity reactions, *Erwinia amylovora*, from apricot twigs, and *Xanthomonas campestris* var. *armoraciae* (McCulloch) Burkholder were not lysed. In a later publication Thornberry *et al.* (30) concluded from cross-lysis experiments using this phage and a variety of test cultures that the phage reaction was more specific than were cross-agglutination tests. Of 212 strains of *Xanthomonas* spp. tested against a mixture of six phage types for *X. pruni* by Eisenstark and Bernstein (8), 13 of the 16 strains of *X. pruni* tested were lysed. Along similar lines of

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experimentation James and Roslycky (17) reported species specificity by 20 phages isolated against strains of *Xanthomonas trifolii* (Huss) comb. nov. (16). Composite filtrates of these phages failed to lyse strains of *Xanthomonas translucens*, *Xanthomonas carolae* (Kendrick) Dowson, *Xanthomonas campestris* (Pammel) Dowson, and diverse other bacteria. Progress has been reported by Katznelson and colleagues (18, 19, 20, 26) in detecting certain seed-borne phytopathogenic bacteria by means of a specific phage plaque-count technique.

Obviously, the potential importance of employing phagic action as a taxonomic tool in differentiating between closely related soil bacteria has both theoretical and practical significance. With particular reference to grouping strains of *Rhizobium* spp., Conn *et al.* (5) in 1945 proposed the value of the bacteriophage in classifying certain soil bacteria. Based on cross-lysis tests employing 22 phages and 33 cultures representing six cross-inoculation groups, these investigators defined four groups of rhizobia: (a) clover, pea, and bean; (b) alfalfa; (c) soybean; and (d) lima bean. Although the groups of rhizobia established by the lytic tests were less exclusive than by cross-inoculation, the lines of division defined by the two sets of criteria were somewhat parallel.

In a recent paper the authors (4) reported the isolation and identification of four rhizobiophages active against *Lotus* rhizobia. Two of these phages were described in moderate detail. The results reported here appraise the host specificities of these four phage isolates in tests against diverse closely related bacteria. Because the *Lotus* rhizobia are similar culturally and physiologically to other *Rhizobium* spp. and yet comprise a closely interrelated group as judged by infective and effective abilities, this experimental approach seemed particularly inviting.

### Materials and Methods

The four races of bacteriophage were isolated from suspensions of plant and root material obtained from the rhizosphere of 4-5-year-old plants of bird's-foot trefoil (*Lotus corniculatus* L.) growing on the East Hill Farm of the local campus. This species had been cultivated in this area for 9 years.

Three criteria were used in the isolation and purification techniques. Initially the crude lysates were filtered through Berkefeld N candles and plated by the double agar-layer technique in serial dilutions against selected susceptible strains of *Lotus* rhizobia. Following incubation, transfers from the centers of well-defined plaques were made into 48-hour broth cultures of the homologous strains. The lysates were again plaqued upon the recurrence of lysis. This procedure with each phage isolate was repeated 10 or more times. Secondly, the isolates were studied in cross-lysis tests similar to those used by Kleczkowska (22) in separating four pure races of pea rhizobiophage from a crude mixture. Thirdly, the isolates which met the afore-mentioned criteria of purity and constancy of characteristics were then screened in broth lysis tests and also by the agar spot method against diverse *Lotus* rhizobia so as to ascertain preliminary host differentiations. As was judged by these criteria, the four phages, P-2, P-3, P-6, and P-11, described here were pure and different.



The quantities of *Lotus* phages required in the following experiments were prepared by adding 1 ml. amounts of each filtrate to 100 ml. amounts of the host organism. After lysis had occurred, the phage-host mixture was filtered through a Selas 03 candle, dispensed into sterile tubes, and stored at 10° C. Each filtrate was assayed prior to use and all portions were equalized to titers approximating  $10^8$  particles per milliliter prior to use. All serial dilutions of the phages were made in 0.1% peptone water.

The bacterial organisms used as hosts in the lytic tests comprised a variety of bacteria. One group consisted of 91 strains of rhizobia obtained from 10 *Lotus* species. Another group of rhizobia represented a broad spectrum comprising 376 rhizobial strains from 105 species of approximately 50 leguminous genera. The diversity of these strains was evidenced not only by their cultural and biochemical reactions, host differences, and geographical range, but also by their diverse infective and effective abilities in relation to their host plants. Still another group of test organisms comprised 40 strains of five species in the genera *Agrobacterium* and *Chromobacterium* (family Rhizobiaceae).

Each strain was tested for lysogenicity by the method of Fisk (12), which was facilitated in time and materials by using large enamel pans covered with glass plates to simulate Petri dishes. Yeast-water mannitol broth, or agar (3), commonly known as Medium 79, modified by the addition of 1.0 g. of granulated potato extract and the substitution of 0.05 g. of calcium chloride for the 3.0 g. of calcium carbonate, served as the medium.

The lytic ability of each phage against the afore-mentioned cultures was tested both in broth and by plaque tests using the double agar-layer technique. In the broth tests 0.1 ml. of the phage filtrate was added to approximately 10 ml. of the rhizobial culture during the early stages of logarithmic growth. Lysis was determined by ascertaining the density of the cultures after 24, 48, and 96 hours with the aid of a Coleman Jr. spectrophotometer. Comparisons were made with lysed cultures of susceptible organisms, identical cultures of the same growth period without phage, and other tubes containing only sterile broth. The plaque tests were made by adding 0.1 ml. of each phage to the surface layer of agar in a Petri dish containing a 12-24 hour culture of the rhizobial strain as predetermined by the relative growth rates of the strains under study. Plaques were discernible usually within an additional 24 to 48 hours' incubation period at 25° C. Final readings were made after 72 hours.

### Results

At the outset of this study each phage was propagated on strain 862, which was originally isolated from a nodule of *Lotus corniculatus* L. In preparation for the host-specificity tests it was deemed desirable to ascertain whether other susceptible host organisms effected phage identification and propagation. Four strains of *Lotus* rhizobia susceptible to lysis by these phages were selected: *L. americanus* 912, *L. angustissimus* 915, *L. lamprocarpus* 929, and *L. suaveolens*

920. After 10 successive transfers of individual phages on each of these strains, a filtrate of the respective phage-host systems and also a filtrate of each phage with *L. corniculatus* 862 were used for comparative purposes in cross-lysis tests against a collection of 10 *Lotus* strains.

Each phage isolate maintained its identity except for slight differences in titer. Host-specificity reactions remained constant. Relatedly, Alexander (1) observed in a critical study of seven phages of *Streptomyces griseus* that each phage retained its specific characteristics regardless of the host strain used for its propagation. Moreover, propagation of the phage on the heterologous strains did not bring about a change in the bacterial reaction with antiserum; the *k* values remained the same. Neither did any phage used by her lose its power to infect the homologous host after a number of serial passages on a heterologous host. In consequence, and for the sake of manipulation, the quantities of each *Lotus* phage used in subsequent host-specificity tests were propagated on *L. corniculatus* 862. This strain, as host, afforded the advantages of rapid growth and minimum gum formation and was markedly susceptible to phage action. Titters approximating  $10^8$  phage particles per milliliter with this organism were obtained without difficulty.

#### *Lytic Tests with Lotus Rhizobia*

As one peruses the reactions of the 91 *Lotus* rhizobia used as hosts (Table I), it becomes obvious that they varied widely in their susceptibility to the phages used. With the exception of the five strains of *L. angustissimus*, none of the groups of organisms *in toto* from any one species was lysed by all the phages; although all the strains from each species with the exception of those from *L. uliginosus* were lysed by at least one of them. Notably, none of the strains from *L. uliginosus* was lysed by any of the phages tested. An explanation for the non-susceptibility of these last-named strains is not known at this time. While it is true that each of these 18 isolates is moderately to

TABLE I  
HOST-SPECIFICITY REACTIONS OF *Lotus* RHIZOBIA

Host plant source	No. of strains tested	No. of strains lysed by phages:			
		P-2	P-3	P-6	P-11
<i>L. corniculatus</i> L.	17	17	11	5	15
<i>L. corniculatus</i> L. var. <i>arvensis</i> Ser.	8	8	8	2	5
<i>L. corniculatus</i> L. var. <i>tenuifolius</i> L.	10	10	8	2	10
<i>L. americanus</i> (Nutt.) Bisch.	7	7	2	0	6
<i>L. angustissimus</i> L.	5	5	5	5	5
<i>L. lamprocarpus</i> Boiss.	8	5	6	2	8
<i>L. ornithopodoides</i> L.	5	5	2	0	5
<i>L. suaveolens</i> Pers.	7	7	7	2	7
<i>L. australis</i> Andr.	6	6	0	2	3
<i>L. uliginosus</i> Schkuhr	18	0	0	0	0
No. of strains tested	91				
No. of strains lysed		70	49	20	64
No. of strains not lysed		21	42	71	27

highly effective on *L. uliginosus*, but all are ineffective on *L. corniculatus*, this coincidence in phage resistance is considered here more circumstantial than correlative. Of further interest was the observation that none of the strains from *L. americanus* and *L. ornithopodoides* was lysed by phage P-6; similarly, none from *L. australis* was lysed by phage P-3. Within the limits of our present knowledge, no particular characteristic of these strains could be attributed to susceptibility or resistance to phagic action.

The lytic range of the phages also showed an interesting diversity in host affinities. Numerically, phage P-2 showed the widest range by its ability to lyse 70 of the 91 test strains. Excluding the 18 strains from *L. uliginosus*, this phage lysed all of the other strains with the exception of three from *L. lamprocarpus*; 64 strains were lysed by phage P-11. Included here were all the strains from *L. corniculatus* var. *tenuifolius*, *L. angustissimus*, *L. lamprocarpus*, *L. ornithopodoides*, and *L. suaveolens*. The narrowest range of lytic ability was shown by phage P-6. The host range of P-3 was also somewhat restricted.

#### *Tests with Other Rhizobia and Closely Related Bacteria*

These organisms comprised two groups of bacteria related in general taxonomic principles to the *Lotus* rhizobia, yet divergent in cultural and biochemical reactions, sources of isolation, and in ecological and host plant relationships. In the first group were 174 strains representative of the presently recognized species of the genus *Rhizobium*, namely: 38 strains of *R. meliloti* Dangeard from 10 plant species in the alfalfa cross-inoculation group, 54 strains of *R. trifolii* Dangeard from 18 clover species, 37 strains of *R. leguminosarum* Frank *emend.* Baldwin and Fred from 14 species, 27 strains of *R. japonicum* (Kirchner) Buchanan, eight strains of *R. phaseoli* Dangeard, and 10 strains of *R. lupini* (Schroeter) Eckhardt, Baldwin, and Fred from six species of *Lupinus*. In addition were 51 strains from 37 species representing 20 plant genera now included in the cowpea cross-inoculation group. One hundred and fifty-one other rhizobial strains comprised isolations from one or more species of the following leguminous genera: *Alysicarpus*, *Anagyris*, *Anthyllis*, *Astragalus*, *Baptisia*, *Caragana*, *Cicer*, *Clitoria*, *Colutea*, *Coronilla*, *Dioclea*, *Galactia*, *Leucaena*, *Onobrychis*, *Ornithopus*, *Petalostemum*, *Robinia*, *Samanea*, *Sesbania*, *Sophora*, and *Tephrosia*. In the second group were included 40 non-rhizobial strains of the genera *Agrobacterium* and *Chromobacterium* (family Rhizobiaceae). These included eight strains of *Agrobacterium radiobacter* (Beijerinck and van Delden) Conn, 16 strains of *A. tumefaciens* (Smith and Townsend) Conn, six strains of *A. rubi* (Hildebrand) Starr and Weiss, and five strains of *A. rhizogenes* (Riker *et al.*) Conn and five strains of *Chromobacterium violaceum* (Schroeter) Bergonzini. *In toto* these two groups of bacteria comprised 416 strains.

Of the rhizobial collection of 376 strains of bacteria, only 28 strains contained in six plant genera, or about 7.5%, were lysed by one or more of the *Lotus* phages. Of special interest was the observation that lysis of the bacterial cultures of *Agrobacterium* and *Chromobacterium* spp. by the phages used did not occur.

TABLE II  
REACTIONS OF *Lotus* RHIZOBIOPHAGES AGAINST RELATED SUSCEPTIBLE RHIZOBIA

Host plant and strain number	Source	Lytic action of phage:			
		P-2	P-3	P-6	P-11
<i>Caragana arborescens</i> Lam., 1030, 1031	Isolated from a shrub growing on the University of Wisconsin campus				
<i>Anthyllis vulneraria</i> L., 11, 12, 13, 14					
<i>Dorycnium rectum</i> Ser., 56, 61, 72, 74		+	+	+	-
<i>Melilotus alba</i> Desr., 122	Collection of H. W. Reuszer, Purdue University, West Lafayette, Ind.	+	-	-	-
<i>Robinia pseudo-acacia</i> L., 755					
<i>Caragana arborescens</i> Lam., 728	Collection of L. T. Leonard, U.S.D.A., Washington, D.C.				
<i>Caragana arborescens</i> Lam., 729					
<i>Astragalus toxicus</i> 1061	Shrub growing near Athens, Ohio				
<i>Astragalus membranaceus</i> Bunge, 1062	Collection of J. K. Wilson, Cornell University, Ithaca, N.Y.	+	-	-	+
<i>Astragalus vernus</i> Oliver, 1057	Collection of J. K. Wilson, Cornell University, Ithaca, N.Y.				
<i>Astragalus wootoni</i> Sheldon, 1065, 1067	Collection of J. K. Wilson, Cornell University, Ithaca, N.Y.				
<i>Caragana arborescens</i> Lam., 725, 726, 727	Isolated from a shrub on the University of Wisconsin campus	+	+	-	+
<i>Caragana arborescens</i> Lam., 1032, 1033, 1034					
<i>Caragana arborescens</i> Lam., 1035	Beaver Lodge Experimental Station, Alta., Canada	+	-	+	-
<i>Caragana arborescens</i> Lam., 1036, 1037	The Nitragin Company, Milwaukee 9, Wis.	+	-	+	+
No. of strains lysed		28	16	13	15

A profile summary of the lytic reactions obtained with these 28 susceptible strains of rhizobia is shown in Table II. All of the strains from *Caragana arborescens* and *Astragalus* spp. were lysed by one or more of the four phage isolates. Ten of the 13 strains of the former species and all of the susceptible *Astragalus* strains were lysed by both phages P-2 and P-11. Such uniformity in phage susceptibility was not obtained with the isolates from any of the other plant genera. Of the six and seven strains from *Anthyllis vulneraria* and *Dorycnium rectum*, respectively, only four strains of each of these plants were susceptible to one or more of the phages used. Of the 38 strains of *Rhizobium meliloti* tested, only one of the four isolated from *Melilotus alba* was susceptible. Strain 755, isolated from *Robinia pseudo-acacia*, was the only strain of this species tested.

Against this group of 28 susceptible rhizobia, phage P-2 showed the maximum host range as contrasted with isolate P-6, which lysed only 13 strains of the group.

### Discussion

The main purpose of this study was to appraise the specificities of four *Lotus* phages in tests consisting of homologous and heterologous rhizobial hosts and closely related bacteria. While, admittedly, the host-specificity reactions of the four phages studied were not as selective as might have been desired, certain interesting parallelisms in the host-phage relationship were obtained. Firstly, each phage isolate exhibited a well-defined range in lytic specificity for the *Lotus* rhizobia used, although no one phage lysed all of the *Lotus* strains. None of the strains of rhizobia isolated from *L. uliginosus* was lysed by any of the lytic agents. Secondly, of 376 strains of related rhizobia obtained from diverse plant host and ecological sources, 28 strains from plants in six genera, or about 7.5%, were lysed by one or more of the *Lotus* phages. Within the limits of our knowledge, any distinctive characteristic that might explain the susceptibilities of these strains is unknown. Thirdly, none of the phage isolates lysed any of the closely related non-rhizobial strains.

Although the studies reported here were exploratory and somewhat limited in scope, it appears logical to reserve optimism that rhizobiophages hold exceptional promise as effective agents in segregating rhizobia into species categories as they are now established. Reasons for this view stem directly from an analysis of the present taxonomic pattern of the genus, *Rhizobium*, which defines species differentiation primarily on the ability of strains to nodulate certain leguminous plants but not others. Distinctive and practical as this criterion of cross-inoculation has been in past years, it is well to remember that nodule formation is an end point reaction of both host and invading strain and can hardly be considered a sole characteristic of either the macro- or the micro-symbiont (2, 25). Within recent years three broad divergencies from the original concept of cross-inoculation grouping have been well established: (a) the lack of mutual interchangeability among strains of rhizobia isolated from plants within certain groups, (b) infective promiscuity of rhizobia from plants within some groups for plants in other ones, and (c) non-reciprocal infective ability of various strains. Thus, in the light of the current confusion regarding validity of infectiveness as a taxonomic criterion for *Rhizobium* spp., it is postulated that any species-specific phages would likely be more sporadic than conventional. On the other hand, these findings support in principle the differentiation of rhizobia from closely related bacteria by phagic action.

Unfortunately, studies on the host-specificity reactions of rhizobiophages are few and have been somewhat restricted in diversification of strains and lytic agents used. The practical value of phagic differentiation of the rhizobia awaits, therefore, further assessment.



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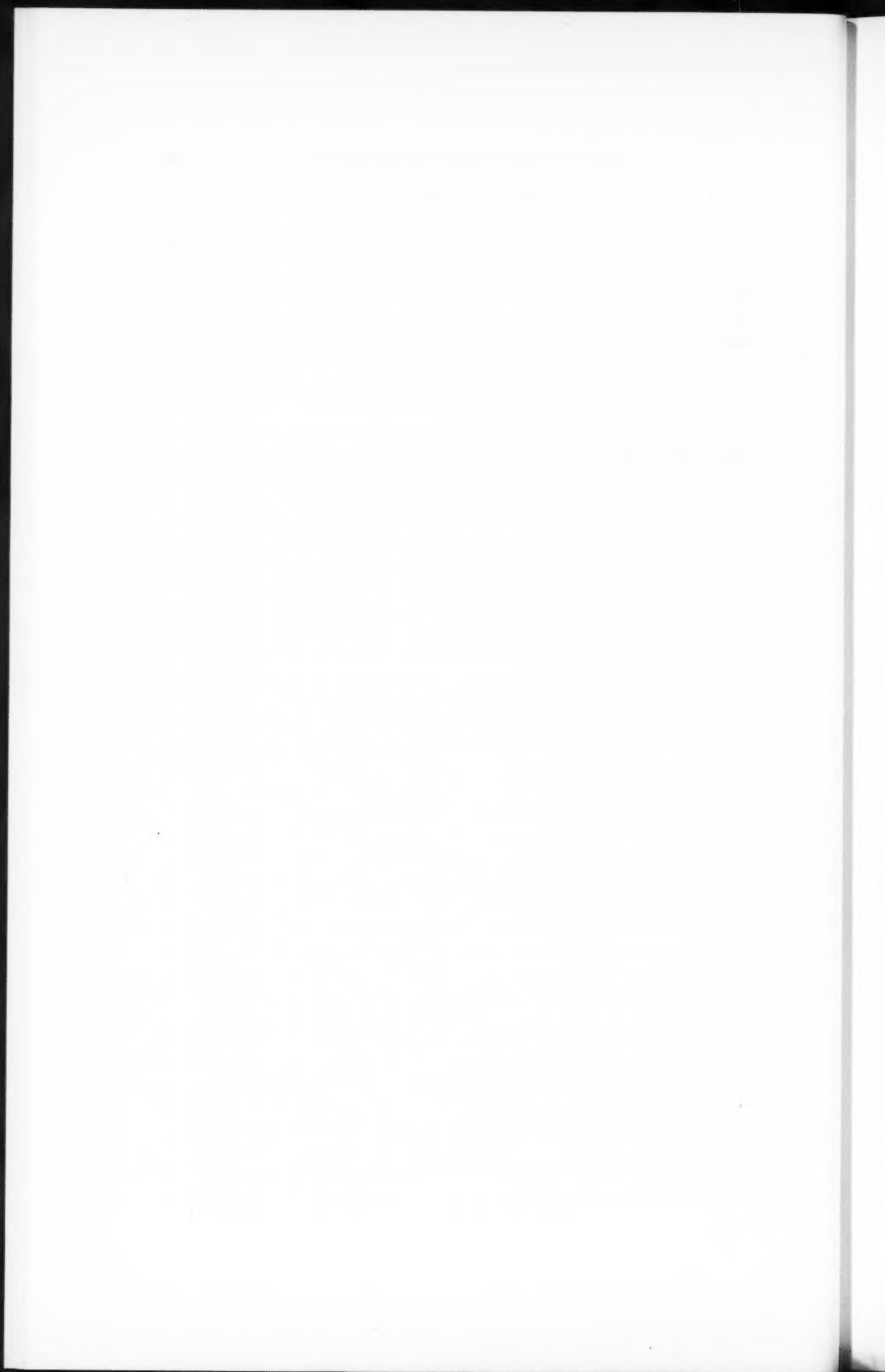
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## CORRELATION BETWEEN BACTERIAL NUMBERS AND CARBON DIOXIDE IN A FIELD SOIL<sup>1</sup>

P. H. H. GRAY AND R. H. WALLACE

### Abstract

Bacterial numbers, estimated by the plate method, and carbon dioxide were significantly correlated ( $r = 0.50-0.69$ ) in field soil during 1955, in an experiment designed to test the effects of straw and crop residues on soil conditions. Differences in moisture, temperature, and soil treatment did not interfere with the correlation.

Since bacteria in soil are dependent upon available energy-yielding material, and since also the evolution of carbon dioxide by the whole microbial population is dependent upon the same material, it seems reasonable to expect to find some degree of direct relationship between the numbers of bacteria in that population and the results of their activities, expressed as the rate of carbon dioxide produced. This is assuming, however, that the bacteria in an established population produce a consistent fraction of the total carbon dioxide evolved. Laboratory experiments in which decomposable organic matter has been added to soil have usually shown that the most rapid evolution of carbon dioxide precedes the development of new bacterial cells. Experiments such as these, however, do not adequately represent conditions in either virgin or field soils.

Very little is known about the microbial ecology in virgin soils in relation to respiration. Gray and McMaster (2) and Gray and Taylor (3) estimated bacterial numbers by the plate method and determined the evolution of carbon dioxide in separate horizons of four virgin podsol soils of Quebec. The correlation coefficient between bacterial numbers and carbon dioxide in samples from the nine lower horizons of those soils was at the 98-99% level of significance (unpublished data). In the samples from the four A<sub>1</sub> (organic matter) horizons the carbon dioxide was closely correlated with the organic carbon ( $r = 0.994$ ) but the bacteria were less significantly correlated with it ( $r = 0.705$ ). The bacterial numbers in these four samples were not correlated with the carbon dioxide. Many more analyses of samples from virgin soils are needed to confirm or refute these close relationships in the surface horizons, in which the bacteria may be in a less stable condition than they are in the lower horizons.

Jensen (4) studied 50 Australian field soils that had varying amounts of organic matter. Estimates of the numbers of bacteria were derived from sets of plates which conformed to a normal Poisson distribution. He found that there was a highly significant correlation between the numbers of bacteria and the organic matter; he did not, apparently, determine the carbon dioxide

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evolution from those samples. In 1936 he presented evidence (5) of an association in 13 soils between carbon dioxide evolution and bacterial numbers estimated by the ratio method of Thornton and Gray (7). An analysis of his data shows that carbon dioxide evolved in 10 days from these soils kept at 15° C. was significantly correlated with the numbers of bacterial cells; and that each of these variables estimated at the start of the experiment was also significantly correlated with the organic matter of the soils. Jensen noted that bacterial numbers (estimated by the plate method with dextrose-casein agar) were not correlated either with organic matter or with carbon dioxide.

This paper presents an interesting example of correlation between bacterial numbers, estimated by the plate method, and carbon dioxide evolved from samples of a field soil. The results are part of those obtained from a long-term experiment now being undertaken in co-operation with the Department of Agronomy, Macdonald College, in which an attempt is being made to ascertain if plant material, plowed in annually, may serve to rebuild depleted organic matter in light soils that have been overcropped or eroded, and to what extent some groups of bacteria may be associated with that rebuilding.

### Experimental

In the field experiment, four replicate adjacent areas, on Chicot sandy loam at Macdonald College, are each divided into five randomized plots. One plot in each area is kept fallow and free of weeds as a control; the other four plots are planted each year with annual sweet clover, and with oats as a nurse crop. One of the latter plots receives chopped straw, one receives superphosphate, one straw and superphosphate, and one no treatment, before sowing. In addition, all of the plant material grown on each plot is plowed in at the end of the season. Preliminary tests indicated that the whole area was uniform in respect to numbers of bacteria and actinomycetes, estimated by the plate method, and to the evolution of carbon dioxide determined by the aspiration method.

The experiment was started in the spring of 1953. During 1955 samples of soil were collected on two dates in June from each of the 20 plots; in July (on one date) and in August (on two dates) composite samples were made from the four plots of each treatment. Soil samples were taken by means of a Lamotte sampling tube to a depth of 6 in., to provide enough soil for estimating bacterial numbers, for carbon dioxide evolution, and for moisture content. For bacteria, with actinomycetes, five plates of glucose-nitrate agar were prepared from a 1:100,000 suspension of each sample; the plates were incubated at 20° C. for 10 days. The plating medium was that of Smith and Dawson (6) but modified by the omission of rose bengal. For the evolution of carbon dioxide, 200 g. of fresh soil were placed in a 1-liter suction flask connected to a series of three tubes of barium hydroxide solution, 0.1 N, in the manner described by Fred and Waksman (1). Aspiration was continuous for 10 days. All results have been expressed on the basis of soil dried by infrared heat.

### Results

The results from the 40 samples taken on two dates in June are shown in Fig. 1. It is clear that the evolution of carbon dioxide was correlated with the numbers of bacteria and actinomycetes. Calculations of the correlation coefficient,  $r$ , indicated that the correlation was highly significant. The relationship between the two variables in the samples taken in July and August, together with the five-plot averages from the samples taken in June, is shown by means of the scatter diagram in Fig. 2. The results from these composite samples, taken together with the averages of those from the first two samplings, show that the correlations between carbon dioxide evolution and bacterial numbers remained significant throughout the season.\* This is

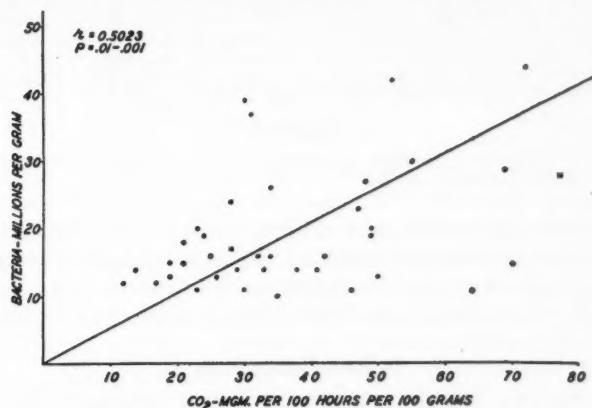


FIG. 1. Correlation between bacterial numbers and carbon dioxide for samples taken in June.

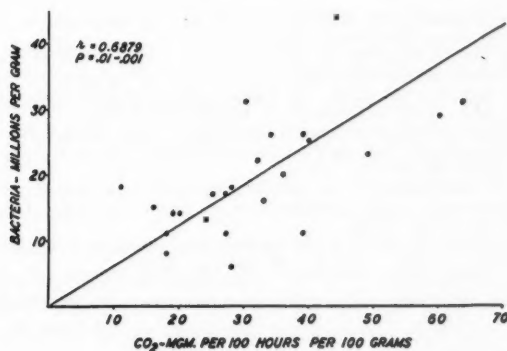


FIG. 2. Correlation between bacterial numbers and carbon dioxide for composite samples taken five times during the summer, including those of June.

\*It may be necessary to point out that only about 50% of the total variability has been accounted for in this analysis.

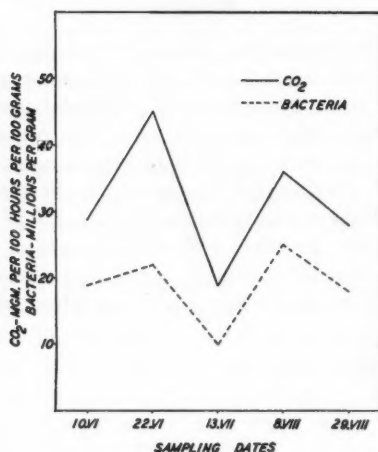


FIG. 3. Fluctuations of bacterial numbers and carbon dioxide in composite samples taken at intervals during the summer.

particularly interesting since both of these variables fluctuated considerably from one sampling to the next, as shown in Fig. 3. It may be concluded that differences in moisture, time, temperature, soil treatment, and cropping were not sufficient to weaken the correlation between these two variables.

### Acknowledgment

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## MICROBIOLOGICAL FORMATION OF VITAMIN B<sub>12</sub>

### II. SYNTHESIS OF VITAMIN B<sub>12</sub>-ACTIVE SUBSTANCES BY BACTERIA ISOLATED FROM CLAMS<sup>1</sup>

B. A. SOUTHCOTT AND H. L. A. TARR

#### Abstract

Vitamin B<sub>12</sub><sup>2</sup> occurred in certain seaweeds and this vitamin and other cobalamins were demonstrated regularly in marine invertebrates. A *Bacillus* and a *Micrococcus* isolated from fresh clams were selected for detailed study on the basis of their pronounced ability to form cobalamins, and because they appeared to be typical of the bacterial flora of these invertebrates. The *Bacillus* formed vitamin B<sub>12</sub> and other cobalamins, and this activity was stimulated by addition of Factor B and of certain purines or benzimidazoles to the medium. With both organisms, 6-nitrobenzimidazole proved particularly effective in stimulating formation of both vitamin B<sub>12</sub> and of other cobalamins, and Factor B stimulated this activity. Formation of cobalamins was associated with a marked decrease in Factor B. The cobalamins were associated with the bacterial cells, and no cobalamin synthesis by washed cell suspensions in phosphate buffer was observed.

#### Introduction

In previous investigations carried out at this Station it was shown that clams (26) and other marine invertebrates (20) were usually good sources of vitamin B<sub>12</sub>, and that this vitamin was readily formed by growth of certain streptomycetes in fish press liquid (24). At that time it was known that vitamin B<sub>12</sub> was a single compound (cyanocobalamin), and one in which the co-ordinatively bound cyano group was replaceable by other ions or molecules with formation of modifications such as hydroxo-, nitro-, and thiocyanato-cobalamins (4, 19). Subsequent work, based largely on chromatographic and electrophoretic separations, and also on the different responses of microorganisms and of animals, indicated that a large number of different cobalamins occur naturally (5, 7, 9, 11, 15, 17, 21). Proof of the structure of vitamin B<sub>12</sub> (2, 14) finally led to the conclusion that, while the vitamin itself contained the "nucleotide-like" residue 5,6-dimethyl-1-( $\alpha$ -D-ribofuranosyl)-benzimidazole-3'-phosphate (1, 18), substitution of this nucleotide by certain other benzimidazoles or purines would result in formation of a variety of different cobalamins (1, 7, 9, 11, 21). So far this substitution has only been accomplished microbiologically by incubation of naturally occurring (11, 12) or chemically prepared (1) Factor B, the nucleotide-free residue of vitamin B<sub>12</sub>, with certain microorganisms in the presence of various benzimidazoles or purines (11, 21). In a few instances the compounds formed have been isolated, purified, and chemically identified, but in most cases this has not been accomplished, the evidence for their presence being based purely on separation by paper chromatography or zone electrophoresis on filter paper, followed by development of "bioautographs".

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<sup>2</sup>Cobalamin in which the nucleotide residue is 5,6-dimethyl-1-( $\alpha$ -D-ribofuranosyl)-benzimidazole-3'-phosphate.

The present work was undertaken to ascertain whether different cobalamins occur in marine invertebrates and in seaweeds, and the possible role of the natural bacterial flora of these marine forms on the synthesis of cobalamins. A brief report of preliminary studies has been published (23).

### Materials and Methods

#### *Chemical Preparations*

The following compounds were used: riboflavin, adenosine-5'-phosphate, adenine, xanthine, guanine, thymine, uracil, ribonucleic acid (Nutritional Biochemicals Corporation); vitamin B<sub>12</sub> (cyanocobalamin) (Merck & Co. and Nutritional Biochemicals); vitamin B<sub>12a</sub> (hydroxocobalamin (Lederle Laboratories); vitamin B<sub>12c</sub> (nitrocobalamin, prepared in this laboratory, see Smith (22)); 1,2-diamino-4,5-dimethylbenzene and 5,6-dimethylbenzimidazole (Hoffmann-La Roche and Merck & Co.); pseudovitamin B<sub>12</sub> (Parke, Davis & Company); benzimidazole, 6-nitrobenzimidazole, 2-amino-benzimidazole, benzthiazole (Eastman Kodak); 2-thioadenine and 2,6-diaminopurine (California Foundation for Biochemical Research).

Factor B was first prepared from calf faeces (12) and was later purified by zone electrophoresis (15). It is regarded chemically as cyanocobalamin from which the nucleotide has been removed and is, presumably, the amide corresponding to the hexacarboxylic acid (1, 2) which has been prepared from vitamin B<sub>12</sub>. So far it has apparently not been obtained as an analytically pure crystalline compound. In the present work this factor was prepared chemically. In a typical experiment, 10 mg. of crystalline vitamin B<sub>12</sub> (Merck) was dissolved in 2 ml. of 70% perchloric acid and heated 4 minutes in a water bath at 60–65° C. The solution was cooled promptly, diluted with 3 ml. of water, and an excess of anhydrous sodium sulphate added. All the colored components of the mixture were extracted with several successive small portions of benzyl alcohol, and these were then displaced into a very small volume of water by adding two volumes of diethyl ether to the benzyl alcohol extract and shaking the mixture in a separatory funnel. The watery solution was evaporated to a very small volume in a rotary evaporator at 37° C. An electrophoretically and chromatographically homogenous preparation of Factor B was obtained by successive zone electrophoresis in 0.05 *M* phosphate buffer pH 6.5 containing 0.01% potassium cyanide (1), and in 0.05 *M* acetic acid containing 0.01% potassium cyanide (15); and finally by continuous paper electrophoresis (8) in 0.05 *M* acetic acid. The final eluate was concentrated to 10 ml. The absorption spectrum of the cyanide-free form exhibited maxima at 354, 498, and 528 mμ, and that of the dicyano form at 367, 540, and 580 mμ (1), and both spectra were similar to these shown by Gregory and Holdsworth (13). A yield of 4.85 mg. of Factor B was obtained as calculated from the optical density of a 1 in 10 dilution of the dicyano form at 367 mμ using the usual  $E_{1\%}^{1\text{cm}} = 210$  value of cyanocobalamin as an arbitrary standard (13). Factor B solutions in 0.01% potassium cyanide were stored at 0° C. in the dark.

### Chromatography

A number of different solvent systems which have been suggested in the literature for separation of cobalamins by paper chromatography were used in initial work. A secondary butanol - acetic acid - water - cyanide solvent (13) proved most satisfactory. Control aqueous solutions of cobalamins containing 1  $\mu\text{g.}/\text{ml.}$  were prepared and 5  $\mu\text{l.}$  spots were applied to Whatman No. 1 paper. Since many of the bacterial cell extracts (*vide infra*) contained comparatively low concentrations of cobalamins, it was found necessary to apply six successive 5  $\mu\text{l.}$  portions to the paper with intermediate drying. The cobalamins were separated by descending development for 18 hours at 37° C. Though electrophoretic separation (15) was used in early experiments, it was found somewhat more cumbersome and did not yield more useful information than did paper chromatography.

### Bioautographs

The medium employed and the conditions were similar to those described for a pad-plate assay for vitamin B<sub>12</sub> (10, 16). One milliliter of a 16-hour vigorous broth culture of the *Escherichia coli* mutant 113-3 was used to inoculate 300-ml. portions of the molten assay medium which was then permitted to solidify in 30 X 45 cm. Pyrex dishes. The paper chromatograms, from which all solvent had been permitted to evaporate, were applied to the seeded agar. They were removed after 30 minutes, the dishes being incubated for 16-18 hours at 37° C. This method is very sensitive in that visible purple zones are formed where it is difficult to locate bacterial growth in absence of the indicator. The bioautographs developed in this manner were photographed by direct contact printing.

## Results

### Cobalamins in Seaweeds and Marine Invertebrates

In initial studies, numerous extracts were prepared from local seaweeds, clams, and oysters, employing a variety of methods. The results indicated that the seaweeds all contained vitamin B<sub>12</sub> itself in comparatively low concentration. Only in one instance was there an indication of the presence of another form of the vitamin. This may have been because the sensitivity of the assay method employed was inadequate to detect the presence of possible very low concentrations of other cobalamins. On the other hand oysters, clams, and tube worms (*Eudystilia vancouverii*) contained comparatively high concentrations of vitamin B<sub>12</sub> and of other cobalamins. Since clams yielded extracts of higher potency than the other invertebrates, they were used in detailed studies. Fig. 1 shows a bioautograph in which an aqueous, cyanide-containing (pH 6.4) extract of clam (*Saxidomus giganteus*) was compared with vitamin B<sub>12</sub> (cyano-, hydroxo-, and nitro-cobalamins) and pseudovitamin B<sub>12</sub> (7, 11, 17). In this experiment, since the solvent contained cyanide, all the three forms of vitamin B<sub>12</sub> were converted to cyanocobalamin and therefore ran at the same rate, while pseudovitamin B<sub>12</sub> ran much more slowly.

It will be seen that the pseudovitamin B<sub>12</sub> preparation was not completely homogenous, and this verifies electrophoresis experiments (15). The clam extract gave four zones, one of which was apparently cyanocobalamin. Results of a number of other experiments, not reported here for sake of brevity, showed that extracts of different samples of clams (*S. giganteus* and *Paphia staminea*) and of oysters (*Ostrea gigas*) always contained not only vitamin B<sub>12</sub> itself, but also a variety of other cobalamins which migrated more slowly in the solvent system used. At this time the publications of the Reading and Cambridge groups referred to above appeared, and the investigation was modified in order to study the formation of cobalamins by bacteria isolated from clams, and the role of Factor B and of various purines or benziminazoles in this formation.

### Isolation of Bacteria

In general, the bacteria associated with clams appear to differ from those usually found in fish (25). One hundred cultures were obtained from clams by usual plating methods and all were grown in nutrient broth and then screened by the pad-plate method (16) to determine their ability to form vitamin B<sub>12</sub>-active substances. It was found that, on repeated transfer in laboratory media for several weeks, many of the organisms appeared to lose their ability to form vitamin B<sub>12</sub>-active substances, and only two of these

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NOTE: "Numerals" represent origins of the chromatograms. "Controls" indicate application of aqueous solutions of vitamin B<sub>12</sub> or Factor B.

FIG. 1. A comparison of chromatograms prepared from different forms of vitamin B<sub>12</sub>, from pseudovitamin B<sub>12</sub>, and from a typical cyanide treated clam extract.

- |  |  |
|--|--|
| 1. Clam extract                              | 4. Vitamin B <sub>12a</sub> (hydroxocobalamin) |
| 2. Pseudovitamin B <sub>12</sub>             | 5. Vitamin B <sub>12</sub> (cyanocobalamin)    |
| 3. Vitamin B <sub>12c</sub> (nitrocobalamin) |  |

FIG. 2. Cobalamin formation by a *Bacillus* species after 64 hours' incubation in presence of Factor B and the following additives:

- |                       |                                      |
|-----------------------|--------------------------------------|
| 1. Factor B (control) | 5. Glucose                           |
| 2. Factor B + ribose  | 6. Factor B                          |
| 3. Factor B + glucose | 7. Basal medium alone                |
| 4. Ribose             | 8. Vitamin B <sub>12</sub> (control) |

FIG. 3. As in Fig. 2 but with the following additives:

- |                             |                                      |
|-----------------------------|--------------------------------------|
| 1. Factor B (control)       | 5. Factor B + benzthiazole           |
| 2. Factor B + riboflavin    | 6. Factor B + 2-aminobenziminazole   |
| 3. Factor B + 2-thioadenine | 7. Factor B + 6-nitrobenziminazole   |
| 4. Factor B + adenine       | 8. Vitamin B <sub>12</sub> (control) |

FIG. 4. Effect of 6-nitrobenziminazole and other additives on formation of cobalamins by a *Bacillus* species after 24 hours' incubation:

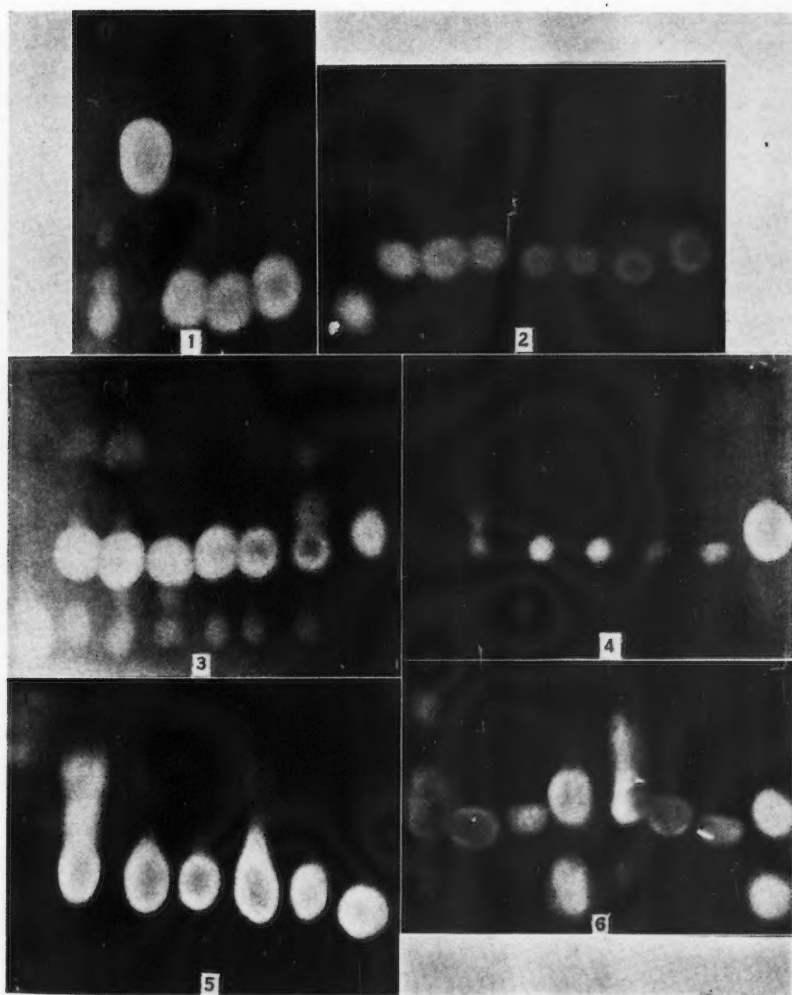
- |  |                                      |
|--|--------------------------------------|
| 1. Factor B (control)                        | 4. Factor B                          |
| 2. Factor B + glucose + 6-nitrobenziminazole | 5. Glucose                           |
| 3. Factor B + glucose                        | 6. Basal medium alone                |
|  | 7. Vitamin B <sub>12</sub> (control) |

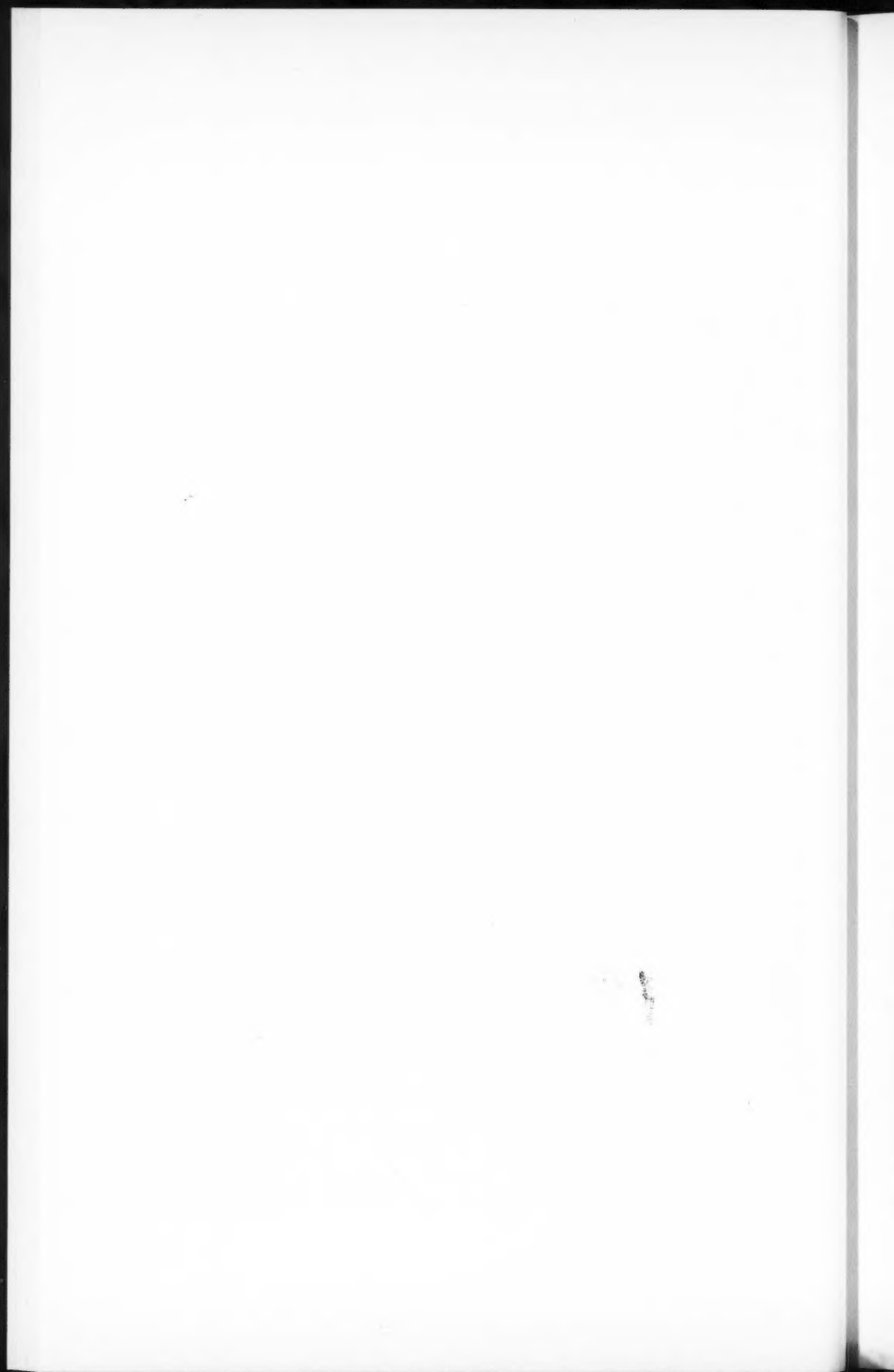
FIG. 5. Effect of 6-nitrobenziminazole on formation of cobalamins by a *Bacillus* species after 144 hours' incubation. Additives as in Fig. 4.

FIG. 6. Effect of 6-nitrobenziminazole on formation of cobalamins by a *Micrococcus* species after 72 hours (Nos. 1-4) and after 192 hours (Nos. 5-6) with the following additives:

- |   |
|---|
| 1 and 5. Factor B + glucose + 6-nitrobenziminazole            |
| 2 and 6. Factor B + glucose                                   |
| 3 and 7. Basal medium   |
| 4 and 8. Vitamin B <sub>12</sub> and factor B (mixed control) |

PLATE I







which proved most active were selected for detailed study. Classification (3) indicated that one of these organisms closely resembled *Micrococcus pyogenes* var. *aureus*, and that the other was a *Bacillus* species.

The formation of cobalamins by these organisms when cultivated in the presence of Factor B and various benzimidazoles or purines was investigated. Still cultivation was used since cultivation on a rotary shaker at 20° or 25° C. failed to increase cobalamin formation. Both organisms grew at 0° and at 10° C., but since growth and cobalamin synthesis was more rapid at 25° C., this temperature was used. It was found that washed cells of the organisms occasioned no apparent cobalamin synthesis in 24 to 72 hours in phosphate buffer, pH 6.7, in the presence of Factor B and 5,6-dimethylbenzimidazole or 1,2-diamino-4,5-dimethylbenzene.

#### *Cobalamin Formation with Bacterial Isolates*

The organisms were grown in 50 ml. portions of Burkholder's medium (6) in 500 ml. conical flasks at 25° C. Factor B and the various compounds were added to the medium in 1 µg./ml. concentration. Since the cobalamin activity was found in the bacterial cells, the organisms were collected by being centrifuged at 6000 g and extracted with hydrochloric acid in the presence of cyanide (11), thereby ensuring that all cobalamins would be in the cyano form. In a number of different experiments it was found that 5,6-dimethylbenzimidazole, riboflavin, adenine, 2-thioadenine, benzimidazole, 2-aminobenzimidazole, and 6-nitrobenzimidazole caused formation of cobalamins which differed chromatographically from vitamin B<sub>12</sub> itself, and that these cobalamins were always formed in greater amount in the presence of these compounds than when Factor B alone was added to the medium. Adenosine-5-phosphate, thymine, guanine, uracil, xanthine, ribonucleic acid, 2,6-diaminopurine, and 2,3-diamino-4,5-dimethylbenzene did not support formation of cobalamins other than vitamin B<sub>12</sub> from Factor B.

Figs. 2 and 3 illustrate the formation of various cobalamins by the *Bacillus* species when cultured 64 hours in both the presence and absence of Factor B and various additives. From Fig. 2 it is apparent that in all instances the organism occasioned disappearance of Factor B with an increase in vitamin B<sub>12</sub>. Small amounts of a slow-moving cobalamin were present in all inoculated media containing Factor B, and even in the medium without any additive. When glucose or ribose was added without Factor B the amount of this slow-moving factor formed was almost negligible. In Fig. 3 are recorded the results obtained by cultivating the *Bacillus* in media containing Factor B plus glucose and six different compounds, and, for these, No. 3 (Fig. 2) should be used as the control. They show that, with the probable exception of benzthiazole, all compounds stimulated formation of cobalamins other than vitamin B<sub>12</sub> with a decrease in the amount of Factor B. With 6-nitrobenzimidazole several different growth zones formed, indicating the presence of several cobalamins.

In view of the above finding, subsequent work with this organism was carried out using 6-nitrobenzimidazole. From Fig. 4 it will be seen that

only when Factor B plus 6-nitrobenziminazole and glucose were added to the medium were significant amounts of cobalamins other than vitamin B<sub>12</sub> formed after 24 hours. After 144 hours (Fig. 5) the picture had altered considerably, for a slow-moving cobalamin was present in several of the media containing Factor B, glucose, or glucose plus Factor B, but the amount of this cobalamin was considerably greater in the medium containing 6-nitrobenziminazole.

In view of the results with the *Bacillus* an experiment was carried out with the *Micrococcus* in which 6-nitrobenziminazole was the only substance investigated. The results, shown in Fig. 6, indicate that only when this compound plus glucose and Factor B were present in the medium were significant amounts of cobalamins other than vitamin B<sub>12</sub> formed. With this organism there was no apparent difference in the approximate amount of cobalamin after 72 and 192 hours.

### Discussion

Observations made in this and in other laboratories have indicated that marine forms of life are usually relatively good sources of cobalamins. Since these are very largely formed in nature by microorganisms, a preliminary study of their occurrence in some seaweeds and marine invertebrates has been made. The results showed that vitamin B<sub>12</sub> itself was present in all forms tested, and moreover, that a large proportion of the cultures which were isolated formed this vitamin. On the other hand, various forms of cobalamins which moved more slowly than did vitamin B<sub>12</sub> itself were present. In the present experiments Factor B was never detected in clam or bacterial cell extracts, and in this respect these materials appear to differ from extracts of calf faeces, rumen contents, and of vitamin B<sub>12</sub>-requiring *E. coli* mutants.

The present work shows that the formation of cobalamins other than vitamin B<sub>12</sub> itself may occur extensively in marine forms of life, and that bacteria isolated from these utilize Factor B in the formation of vitamin B<sub>12</sub> and of other cobalamins. With the organisms studied 6-nitrobenziminazole was found to be the most active of a number of purines and benziminazoles investigated in occasioning formation of vitamin B<sub>12</sub> and other cobalamins from Factor B.

Since the actual quantities of cobalamins formed in experiments such as the above are extremely small, no attempt has yet been made to concentrate or isolate them, and the data available on *R<sub>f</sub>* values are hardly reliable enough in themselves to warrant any attempt at distinction. It is highly probable that a single bacterial growth zone arising from a chromatogram may represent two or even more cobalamins. The mechanism by which benziminazoles or purines combine with Factor B to form cobalamins is not clear. It is possible that the substance is first phosphorylated by a phosphorylase enzyme in the presence of ribose-1-phosphate to form a purine or benziminazole nucleoside which yields the corresponding ribotide with adenosine triphosphate in the presence of a kinase enzyme. Certainly the parent nucleotide of vitamin

B<sub>12</sub> itself forms vitamin B<sub>12</sub> when incubated with *E. coli* and Factor B (11). Apparently nothing is known yet regarding the enzyme mechanism which might be involved in linking the phosphate group of the nucleotides to the aminopropanol residue of Factor B, nor the possible role of  $\beta$ -ribotides in replacing the  $\alpha$ -ribotide occurring in vitamin B<sub>12</sub>.

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THE DEVELOPMENT OF BACTERIAL POPULATIONS IN MILK<sup>1</sup>T. GIBSON AND Y. ABD-EL-MALEK<sup>2</sup>

## Abstract

The dominant bacteria in different classes of milk have been identified by taking samples of the colonies on quantitatively inoculated plates. Many of the bacteria that occur commonly in milk find it a relatively unfavorable medium. In the temperature range 10°–22° C. the organisms that multiply actively are the *Streptococcus lactis* group, a species of *Leuconostoc*, coagulase-negative staphylococci, and Gram-negative rods (chiefly *Alcaligenes viscosus* and fluorescent and non-fluorescent pseudomonads). Plate counts of organisms that resist pasteurization for 30 minutes at 63° C. are unsuitable for demonstrating the multiplication of *Leuconostoc* or *Alcaligenes tolerans*, which show variable and frequently low rates of survival in the heat treatment. In laboratory-pasteurized milk incubated without recontamination, the surviving organisms, unlike the populations of raw milk, do not show distinctly the effects of a differential selection. The only thermophilic organism that was found to produce rapid deterioration of milk is *Bacillus cereus* (including *B. mycoides*).

Many investigations have been made into the problems of excluding bacteria from milk and of controlling the growth of those that gain entry. In this field Johns and Lochhead (8) and Lochhead and Johns (9) have made notable contributions. The types of bacteria that occur in the sources from which milk is contaminated have also received attention, but so far not a great deal of interest has been taken in the qualitative composition of the bacterial populations which develop in milk. The objects of the work discussed in this paper were to make a general survey of the microflora in different classes of milk, and to investigate the modifications produced by growth of the organisms. In raw milk comparatively few types of bacteria were shown to multiply actively, and the composition of the populations changed in a definite direction; in uncontaminated pasteurized milk selective influences were much less evident.

## Materials and Methods

Three main types of raw milk were used: (1) specimens obtained by aseptic milking; (2) milk bottled on the farm, herein designated F samples; and (3) specimens taken from bulks in a storage tank of a pasteurizing plant, designated B samples. The third type was chosen because it invariably contained heavily contaminated supplies. The bulks may have included some milk which had been heat-treated.

The milk was plated (two plates at each dilution) on agar containing 10 g. peptone and 2.5 g. glucose per liter of meat infusion. Each plate inoculated with less than 1 ml. of the sample received an addition of 0.5 ml. sterile skim milk. After incubation for 5–6 days at 30° C. the dominant microflora was

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sampled by transferring to litmus milk every colony on one suitable plate or on duplicate plates or on opposite sectors of a plate. An attempt was made to secure a sample consisting of at least 30 colonies. The pure cultures were identified by methods which previously have been described (1, 2, 3, 4).

In the present paper the tables are simplified by grouping the species if it appears that no essential information is thereby lost. The following explanation indicates the content of some of the groups.

*Streptococcus lactis* group—includes *S. cremoris*.

*Leuconostoc*—signifies *Streptococcus kefir* Migula. *Leuconostoc citrovorum* (Hammer) Hucker and Pederson was rarely detected, only once in the absence of *S. kefir*.

*Staphylococcus*—heat-sensitive cocci mainly derived from the animal body; group I (2). The great majority are coagulase-negative.

*Micrococcus*—saprophytic, mainly thermoduric cocci; groups II and III (2).

Diphtheroid group—heat-sensitive corynebacteria, mainly of animal origin.

*Bacillus subtilis* group—includes *B. pumilus* and *B. licheniformis*.

Miscellaneous—various organisms that appear to be incidental contaminants of milk.

When laboratory pasteurization was applied, milk in cotton-stoppered tubes was held at 63° C. for 30 minutes. Care was taken to have no milk on the side of the tube, and to have the milk level well below the level of water in the bath.

## Results

### *The Dominant Bacteria and the Thermoduric Bacteria in Different Classes of Raw Milk*

An analysis was made of the dominant microflora in 38 samples of raw milk, and also of the thermoduric organisms in the same samples. Table I is a summary of the more essential results afforded by the detailed figures.

In uncontaminated (single-quarter) milk, staphylococci and diphtheroid organisms were the dominant bacteria, one or the other group being the more numerous. By using selective methods several of the quarters could be shown to harbor also a few mastitis streptococci. Thermoduric organisms were uniformly absent. In another series of quarter samples the milk in 10-ml. quantities was pasteurized and then incubated at 22° and at 37° C.; thermoduric bacteria were detected in none.

The single-farm samples (from seven farms) are divided in the table into two classes: (a) a group of five in which the udder organisms were still prominent, and (b) a group of eight samples containing a denser and more complex microflora. In four of (b) *Corynebacterium lacticum* was the dominant organism in the raw milk.

The bulk milk, affected by heavier contamination and more bacterial growth, was characterized by the preponderance of *Streptococcus lactis* and *Leuconostoc*. It is noteworthy that the latter was detected in greater numbers than the *S. lactis* group in all but two of the samples. Another feature of the



TABLE I

THE NUMBER OF SAMPLES OF EACH CLASS OF MILK IN WHICH THE BACTERIAL GROUPS WERE DETECTED

	Uncontaminated milk, 15 samples	Milk from single farms		Bulk milk, 10 samples
		5 samples	8 samples	
Data from plating of raw milk				
<i>Streptococcus lactis</i> group	—	(2)	(5)	10
<i>Streptococcus faecalis</i> group	—	—	(1)	(6)
<i>Streptococcus bovis</i>	—	—	—	(1)
Mastitis streptococci	1*	4	3	8
<i>Leuconostoc</i>	—	(3)	5	9*
<i>Staphylococcus</i>	12*	5*	7	8
<i>Micrococcus</i>	(1)	(2)	4	(1)
Diphtheroid group	7	4	(2)	(4)
<i>Corynebacterium lacticum</i>	—	—	7	(3)
<i>Alcaligenes viscosus</i>	—	3	7	8
<i>Alcaligenes tolerans</i>	—	—	(2)	(2)
Coliform group	—	—	(1)	(4)
<i>Pseudomonas fluorescens</i>	—	1	3	6
Other Gram-negative rods	—	2	4	6
Miscellaneous	—	3	(3)	(2)
Data from plating after laboratory pasteurization				
<i>Streptococcus faecalis</i> group	—	—	—	4
<i>Streptococcus bovis</i>	—	—	—	10
<i>Streptococcus thermophilus</i>	—	—	—	9*
<i>Leuconostoc</i>	—	—	—	(2)
<i>Micrococcus</i>	—	—	3	10
<i>Corynebacterium lacticum</i>	—	—	8*	10*
<i>Alcaligenes tolerans</i>	—	—	—	(1)
<i>Bacillus subtilis</i> group	—	2*	—	—
Miscellaneous	—	1	(1)	—

\* A dominant group in the majority of samples enumerated.

( ) Rarely more than one to two colonies were isolated from the samples enumerated.

bulk milk is that in nine cases the approximate count of *Streptococcus thermophilus* was in the range 2000–50,000/ml. Considering that the minimum temperature for the growth of this organism is about 18° C. (see Table IV) these figures are unaccountably high.

The data in Table I suggest that there is a fairly distinctive pattern in the bacterial populations of milk. Of many diverse contaminants only a comparatively few appear to proliferate actively either in ordinary raw milk or on dairy equipment. The organisms classified in the table as "Miscellaneous" comprised sarcinae, red-pigmented micrococci, lactobacilli, sporeformers other than those of the *Bacillus subtilis* group, *Nocardia*, and *Streptomyces*. Within each group of samples shown in Table I no definite relationship was detected between season and the composition of the dominant microflora.

#### The Multiplication of Bacteria in Raw Milk

The foregoing survey did not afford a precise distinction between the influence of contamination and the results of bacterial multiplication. With the object of investigating the qualitative effects of bacterial growth, eight of

TABLE II  
THE MULTIPLICATION OF BACTERIA IN RAW MILK

	Samples from single farms									
	F6		F9		F10		F13			
	0 hr. count/ml.	24 hr. 22° X factor	0 hr. count/ml.	24 hr. 11° X factor	0 hr. count/ml.	24 hr. 22° X factor	0 hr. count/ml.	24 hr. 13° X factor	0 hr. count/ml.	24 hr. 22° X factor
<i>S. lactis</i> group	420	1,500			500	20,000	8,200	3	8,200	4,300
<i>Leuconostoc</i>	< 420	> 3,100	450		1,300	13,000	8,200	3	8,200	1,000
<i>Staphylococcus</i>	4,600	570	670		500	7,000	8,200		180,000	220
Gram-negative	2,500	1,200	450	4,200	4,000	6,500	33,000	1		
Other bacteria	9,500		7,100		3,800					
Thermophilic:										
<i>S. boris</i>			< 190		< 60	> 40				
<i>Leuconostoc</i>	< 240	> 380	< 190		< 60	> 18				
<i>Micrococcus</i>	480	5	930	1	2,200	4	34,000	1		
<i>C. lacticum</i>	11,000	1	4,500	1	< 60	> 210	< 970			> 1
<i>A. tolerans</i>										
Samples from bulks										
	B5		B6		B7		B10			
	0 hr. count/ml.	24 hr. 11° X factor	0 hr. count/ml.	24 hr. 10° X factor	0 hr. count/ml.	24 hr. 12° X factor	0 hr. count/ml.	24 hr. 13° X factor	0 hr. count/ml.	24 hr. 22° X factor
<i>S. lactis</i> group	210,000	38	50,000	32	20,000	420	170,000	1,100	170,000	68
<i>Leuconostoc</i>	450,000	16	150,000	5	80,000	74	< 12,000	> 670	< 12,000	> 190
<i>Staphylococcus</i>	53,000		10,000		20,000		24,000	580	24,000	170
Gram-negative	340,000	32	100,000	50	580,000	43	130,000		130,000	
Other bacteria	79,000		50,000		80,000		60,000		60,000	
Thermophilic:										
<i>A. tolerans</i>										
Other bacteria	50,000	1	33,000	1	130,000	1	< 200	> 6	< 200	> 3

. Not represented in the sample of colonies; replaced by < if subsequent multiplication was detected.

the samples used in the survey were examined again after being held at specific temperatures. The holding periods and the temperatures employed are given in Table II. In order to bring out the significant results, the table shows for each organism that gave evidence of having multiplied, (a) the approximate count in the fresh milk, and (b) the factor of multiplication during the holding period. Both figures are derived from the total plate count and the proportionate representation of the organism in the sample of colonies examined. Bacterial groups that failed to show growth are included among "Other bacteria" in Table II. An indication of their identity is obtainable from Table I.

The bacteria that gave evidence of multiplication were the *S. lactis* group, *Leuconostoc*, coagulase-negative staphylococci, and Gram-negative rods. The behavior of *S. lactis* at temperatures of 10°–22° C. conforms to much published information on the bacteriology of milk. Of greater interest just now is the finding that *Leuconostoc* may show similar growth rates in milk at the same temperatures. The Gram-negative rods also proliferated actively, especially in the samples that gave at the start low total plate counts. Although their growth was often slower than that of *S. lactis* or *Leuconostoc*, the rods outnumbered each of these at the end of the holding period in all cases but one. *Alcaligenes viscosus* and *Pseudomonas* were the main organisms in the Gram-negative group; *Alcaligenes tolerans* and coliform organisms appeared much less frequently. Of the groups that showed multiplication, the staphylococci generally gave the smallest increases. The bacterial growth in the different samples exhibited variations for which no simple explanation has been found. Possibly several factors were concerned, such as intergroup competition, the growth phases of the organisms at the outset, and the chemical properties of the milk.

Conclusions that may be drawn from these observations are: (1) if raw milk is held between 10° and 22° C. the organisms that proliferate most actively are likely to be *S. lactis*, *Leuconostoc*, Gram-negative rods, and, in the early stages, staphylococci; (2) the greater the proportion of the total microflora that consists of these groups collectively, the greater will have been the effect of growth and the smaller the contribution of contamination in determining the bacterial content. These conclusions are consistent with the findings reported in Table I insofar as the previous history of the milk is known.

The counts of organisms that resisted pasteurization did not show large increases during the holding periods. This indicates that some, at least, of the thermophilic bacteria have poor competitive powers. Nevertheless, as a method of demonstrating growth, the postpasteurization count has a limitation which was brought out in these experiments. The survival rate of the *Leuconostoc* varied from lower than 1 in 50,000 (in F10 after 24 hours at 22° C.) to 1 in 14 (in F6 after incubation). The rate for *A. tolerans* similarly varied from lower than 1 in 2000 (in F9 after 24 hours at 22° C.) to at least 1 in 40 (in F10 after 24 hours at 13° C.). Both organisms have shown a similar variation in tests on other milk. In contrast, the species that possess greater

resistance to heat have been throughout this work much more consistent in their survival of pasteurization. The heat resistance of bacteria is known to vary in the different phases of growth; the variations just described may be of this nature but the data obtained do not afford evidence on the point. A possibility which cannot be excluded is that in *Leuconostoc* and *A. tolerans* there is a strain to strain variation in heat resistance.

#### *The Multiplication of Bacteria in Pasteurized Milk*

The course of bacterial development in pasteurized milk was followed in four instances until the milk was spoiled. Each sample was distributed in 10-ml. quantities in several tubes and then pasteurized. The tubes were incubated at 22° C. and at either 10° or 13° C., and at intervals the contents of a tube were plated. The final examination was made shortly before or at the time visible changes started to appear.

In Table III are shown the results of the early and the final examinations and of any intervening examinations that provide evidence on the course of events. *C. lacticum* and the thermophilic micrococci showed a delayed start followed, in three cases at 13° and 22° C., by multiplication at a rate similar to that of the other organisms. At 10° C. they finally disappeared from the dominant microflora. *A. tolerans* was in no instance detected immediately after the pasteurization, but in three of the trials it proliferated rapidly within 24 hours at 10°-22° C. *S. bovis* and *S. thermophilus*, when they were present, maintained their proportions of the population at 22° C.; they showed no growth at the lower temperatures. *Leuconostoc* tended to appear late,

TABLE III  
THE DEVELOPMENT OF BACTERIA IN PASTEURIZED MILK

Milk	Temp. ° C.	Time, days	Plate count X 10 <sup>-3</sup>	Identifications				
				<i>C. lacticum</i> + <i>Micrococcus</i>	<i>A. tolerans</i>	<i>S. bovis</i> + <i>S. thermophilus</i>	<i>Leuconostoc</i>	<i>B. cereus</i>
F13	22°	0	34	35	.	.	.	.
		1	3,200	1	30	.	.	.
		7	420,000	18	6	.	10	.
B10	10°	7	950	3	45	.	.	.
		21	350,000	.	12	.	9	6
B10	22°	0	7	33	.	1	.	.
		1	360	2	28	.	4	.
		3	440,000	3	22	7	.	.
B11	13°	3	5,800	2	10	.	1	17
		15	96,000	9	10	.	20	.
B11	22°	0	78	7	.	29	.	.
		1	1,200	2	2	38	.	.
		9	390,000	2	12	24	.	.
B12	10°	7	> 100 (spreaders)	.	.	.	.	20
		20	810,000	.	1	.	33	.
B12	22°	0	67	11	.	25	.	.
		1	1,100	4	.	36	.	.
		4	130,000	2	2	31	.	.
B12	10°	9	59,000	15	.	15	.	.
		15	170,000	3	2	.	26	.
		20	140,000	.	.	.	29	.

possibly for the reason that it is largely destroyed by pasteurization. Its development at 10° and not at 22° C. in B11 and B12 suggests a competition between it and the homofermentative streptococci, brought out in these instances by the difference in numerical proportions before growth started. *B. cereus* behaved unlike the other organisms. It was detected only at 10° and 13° C., thus indicating that the greater activity of other species at 22° C. was in some way repressive. In none of the specimens of milk did the bacillus appear in all the 10-ml. quantities incubated at 10° to 13° C. This implies that it was sparsely represented at the start. Whenever *B. cereus* became numerous clotting of the milk occurred soon afterwards.

A feature of this series of tests is the time required by the milk to reach the clotting stage. B11 and B12, representatives of heavily contaminated supplies, and carrying all the types of thermoduric bacteria that occur frequently in milk, were able to remain unclotted for about 9 days at 22° C. and 20 days at 10° C. The observations on *B. cereus* suggest that minority groups, which may be absent from volumes as large as 10 ml., may be more harmful in pasteurized milk than the commoner thermoduric organisms.

#### *Bacterial Populations in Pasteurized Milk Incubated at 17° C. until Spoiling Occurred*

The work last described suggested a wider survey of the organisms that may spoil efficiently pasteurized milk. This has been made using 24 other samples of varying purity. The quantity pasteurized was increased to 60-70 ml. in order to facilitate the development of organisms which might be absent in the 10-ml. volumes previously used. The pasteurized milk was incubated at 17° C. (exceptions noted in Table IV) and, at intervals, 0.5-ml. quantities were examined for clotting by 68% alcohol and on boiling. When both tests became positive the milk was adjudged to have been spoiled. Two culture examinations were made, one immediately after pasteurization, the other when the milk had reached the stage of abnormality. As in the earlier work, two plates at each dilution were incubated for 5-6 days at 30° C. but, in addition, a third plate at each dilution was incubated for 2-3 days at 37° C. in order to suppress *C. lacticum* and most of the micrococci, and so increase the chances of detecting additional organisms.

The results of the analysis immediately after pasteurization need not be detailed. Twelve samples (F19-F30) from seven farms gave plate counts of 2 to 530,000/ml., in four cases < 20/ml. Samples of bulk milk (B13-B24) showed counts of 6500 to 500,000/ml. In neither series were counts related to keeping quality. The original bacterial populations appeared to be qualitatively similar to those in the samples previously discussed. In all the farm samples *C. lacticum* was the dominant organism; micrococci were found in six; other bacteria were only occasionally isolated from plates at 30° C. Sporeformers were detected in several of the F series by plating 1 ml. at 37° C. The samples of bulk milk all contained *C. lacticum*, fewer *Micrococcus*, and still fewer *S. thermophilus*; eight yielded *S. bovis*; other organisms were rarely found.

The results of the final examinations are shown in Table IV where the samples of each series are arranged in the order of their keeping quality. The keeping periods were distinctly long. The examination of F23 was made after 16 days when the milk was still stable to alcohol and to boiling, though it was then abnormal in taste. The figures for identifications shown in the table apply to the samples of colonies taken from plates incubated at 30° C. The results for the 37° C. plates are not presented, but where they afford additional information a plus (+) sign is used in the table to indicate the organism detected.

Several points emerge from the data. Frequently bacteria which had not been isolated in the first examination became a notable fraction of the final population. This applies especially to the *S. faecalis* group, *Leuconostoc*, and *A. tolerans*. Nevertheless there are no indications that the conditions in the milk were markedly selective for any particular group or that any species was specially antagonized by another. The groups that finally dominated the populations varied from sample to sample as if fortuitously.

TABLE IV  
BACTERIAL POPULATIONS IN PASTEURIZED MILK AT THE TIME OF  
DETERIORATION; INCUBATED AT 17° C.\*

	Samples from single farms											
	F23	F24	F21	F29	F30	F19	F26	F25	F27*	F28	F22	F20
Incubation, days	16	14	10	10	10	9	8	7	7	7	7	6
Plate count $\times 10^{-4}$	300	100	150	400	430	230	12	57	150	3.5	0.8	100
Identifications:												
<i>S. faecalis</i> group	.	.	.	34	38	23	.	.	.	.	.	.
<i>S. bovis</i>	.	.	+	.	.	.	.	.	+	.	.	.
<i>Leuconostoc</i>	.	.	27	.	.	.	.	.	1	.	8	.
<i>Micrococcus</i>	.	.	1	.	.	.	.	.	.	.	.	.
<i>C. lacticum</i>	23	.	2	2	6	2	17	31	22	5	.	43
<i>A. tolerans</i>	12	.	5	.	6	2	3	5	.	.	.	.
<i>B. pumilus</i>	.	+	.	.	.	.	.	.	.	3	.	.
<i>B. circulans</i>	.	.	4	.	.	.	.	.	.	.	.	.
<i>B. cereus</i>	.	.	.	.	.	.	+	.	.	19	+	+

	Samples from bulks											
	B17	B16	B15	B18	B22*	B23	B20	B19	B13	B14	B21*	B24
Incubation, days	8	8	7	7	7	7	6	5	5	5	5	4
Plate count $\times 10^{-4}$	410	690	680	120	220	240	370	110	25	10	19	7.7
Identifications:												
<i>S. lactis</i>	.	.	.	.	.	.	.	17	.	.	.	.
<i>S. faecalis</i> group	21	+	46	35	.	21	.	.	.	.	.	.
<i>S. bovis</i>	.	+	.	.	13	.	.	.	+	+	2	.
<i>S. thermophilus</i>	.	.	.	.	9	.	.	.	.	+	3	.
<i>Leuconostoc</i>	2	19	.	.	6	23	31	.	.	10	+	.
<i>Micrococcus</i>	.	.	.	.	.	.	.	.	.	.	4	.
<i>C. lacticum</i>	1	14	.	.	.	2	1	28	15	27	7	4
<i>A. tolerans</i>	7	1	.	.	.	.	3	.	8	3	5	25
<i>B. circulans</i>	.	.	.	.	.	.	.	+	.	.	.	.
<i>B. cereus</i>	.	.	.	+	.	.	.	.	2(+)	+	1(+)	6

\* The incubation temperature was 19° C. for F27 and B22, 18° C. for B21.

+ Demonstrated at a lower dilution than that used for colony sampling; isolated from (a) 30° C. plates on which spreading growth prevented sampling or (b) 37° C. plates.



The organism that had the greatest effect on milk appears to have been *B. cereus*. It or, in F22, its variety *mycoides* developed in the samples that reached the clot-on-boiling stage early and at a time when the plate count was still low. This relation of early clotting and low count is doubtless associated with the vigorous rennin action of the bacillus, but there exists the further possibility, suggested by the data in Tables III and IV, that the growth of *B. cereus* in milk occurs most readily when the multiplication of other bacteria is for some reason restrained. In the examinations immediately after pasteurization, *B. cereus* was detected only in F20 and F21 (on 37° C. plates); it apparently failed to grow in F21.

*S. lactis* seems to have been the main factor in the deterioration of B19. This was the only instance in the whole investigation in which *S. lactis* was detected in laboratory-pasteurized milk. Tests on pure cultures (1) indicate that its survival rate in that treatment must be extremely low.

*S. bovis*, *S. thermophilus*, *C. lacticum*, and the thermoduric micrococci appear to be incapable of spoiling milk rapidly at 17° C. The lowest temperature at which the growth of *S. thermophilus* has been detected in this work is 18° C. (sample B21). The remaining organisms named in Table IV were scarce at the outset, and perhaps there were long delays before they proliferated. Their potential capacity to spoil milk cannot therefore be assessed. The possibility does exist that active minorities were not detected in certain examinations, but the bacteriological evidence indicates that some of the variation in keeping quality was associated with properties of milk additional to the presence after pasteurization of specific bacteria.

### Discussion

This work has provided information which assists in defining the main course of bacterial development in most milk. Of the diverse organisms which are introduced, a large proportion appear to find milk a relatively unsuitable environment. In the temperature range 10°–22° C. the bacteria that show active multiplication are the *S. lactis* group, a species of *Leuconostoc* (*Streptococcus kefir* Migula), coagulase-negative staphylococci, and Gram-negative rods. The most active of the rods are *A. viscosus* and fluorescent and non-fluorescent pseudomonads. The final result, on which the literature affords much evidence, is likely to be the dominance of a single type, the *S. lactis* group. The literature further indicates that the course of events would be similar at a somewhat higher temperature, and that growth would be largely restricted to Gram-negative rods at lower temperatures.

It would thus appear that milk has pronounced selective properties. Every specimen behaves as an enrichment culture. The mechanisms of the selection are not easy to specify. The growth rates of the organisms are certain to differ; milk is known to have inhibitory effects on certain bacteria; some of the organisms that multiply in it have been shown to produce inhibitory substances; as the clotting stage is approached the accumulation of lactic acid is certain to be an important selective factor. Nevertheless, the bacterial

development has features suggesting that it may be determined mainly by the available food supply. The finding that in some specimens of milk the whole population develops more rapidly or reaches a greater density than in others is a strong indication of a variation in nutritive quality. In 1904 Marshall (10) showed that the growth and activity of a streptococcus in milk could be increased as a result of the previous or simultaneous growth of a proteolytic bacterium, and there have been further observations of a similar nature. Orla-Jensen and Jacobsen (11) and others obtained the same effect by adding various substances to milk. In view of the low concentrations in milk of the components of its non-protein nitrogen fraction and the certainty that these substances are required by many of the bacteria, actions such as protein breakdown will inevitably promote growth. A case of this sort has been examined in detail by Pette and Lolkema (12). The opposite effect, the complete exhaustion of milk of some of its minor constituents, could bring bacterial growth to a stop; it could also account for intergroup competition and for the fact that one group does often overgrow another.

Some organisms which may well be regarded as typical of milk do not find it an ideal habitat. Examples are *C. lacticum* and the thermophilic micrococci. Cuthbert, Egdell, and Thomas (5) review work which showed that these bacteria do not multiply readily in raw milk (see also Table II in the present paper), and Williams (13) found a similar behavior when pure cultures were tested in pasteurized aseptic (sterile) milk. The data in Table III of the present work show that active growth in pasteurized milk was delayed until other thermophilic bacteria had started to multiply. The regular occurrence of such organisms in all but the purest milk can be attributed to the finding that they colonize rubber and metal surfaces of dairy equipment. Such environments differ widely from that in milk.

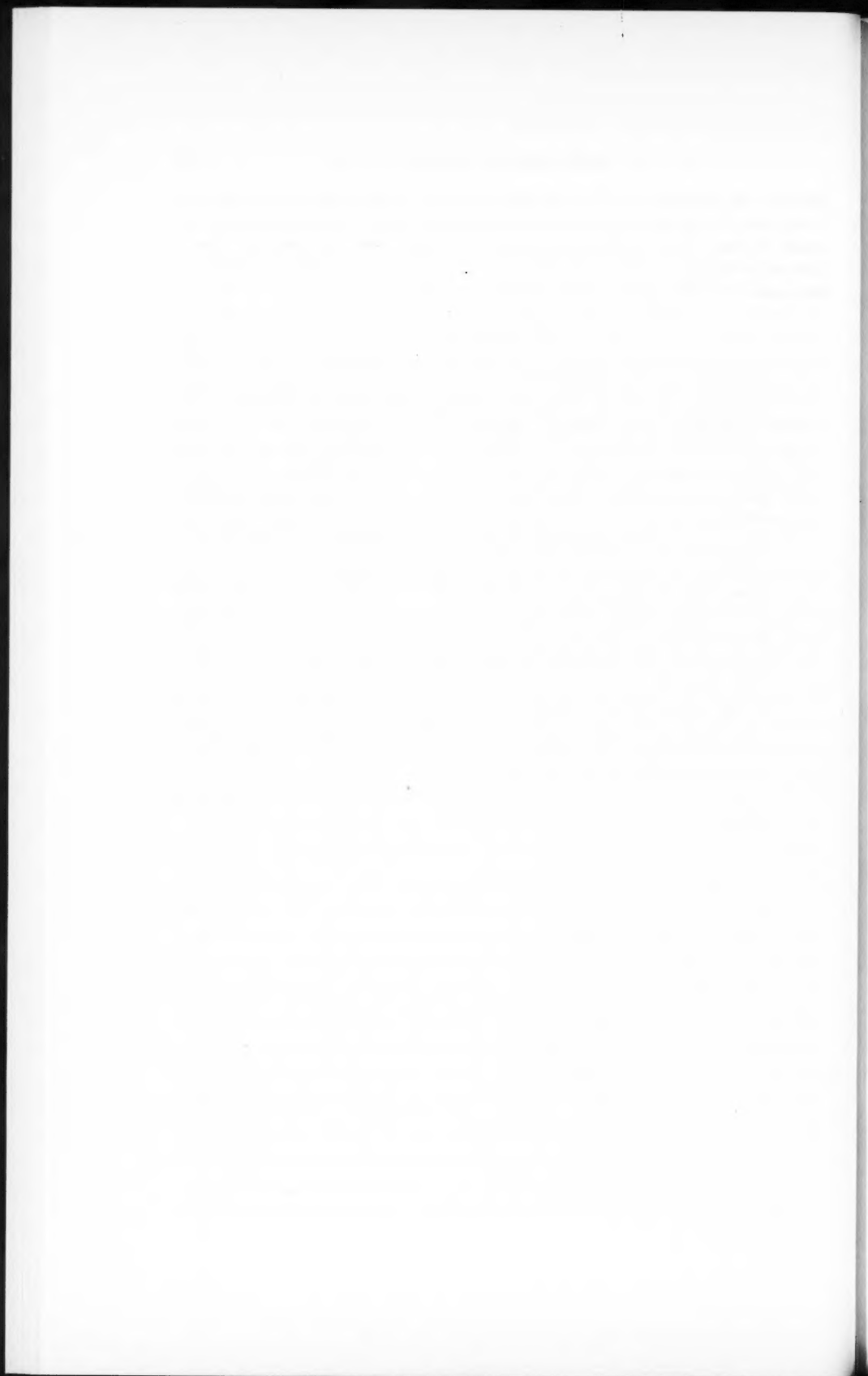
In milk pasteurized in the laboratory for 30 minutes at 63° C. and then incubated without recontamination, the organisms that survived the heating behaved unlike the populations normal to raw milk. The thermophilic bacteria, provided their temperature requirements were satisfied, did not clearly show the effects of a differential selection. In milk pasteurized on a large scale other organisms are commonly reported to develop. Galesloot (7), who made a thorough examination of this question, found that *S. lactis* and Gram-negative rods appeared in most samples held at 15°, 20°, and 27° C. Apparently the sequence of changes tended to approach more or less closely that characteristic of raw milk. It may therefore be concluded that the features of bacterial growth in laboratory-pasteurized milk are determined by the weakness in competitive powers of the thermophilic bacteria. In the present work, Gram-negative rods other than *A. tolerans* were never detected in the incubated pasteurized milk. Where they have been found after pasteurization at 63° C. in the laboratory, the conditions would appear to have been in some respect akin to those in a commercial process.

There is a growing opinion that in laboratory-pasteurized milk the only species capable of inducing a rapid deterioration is *B. cereus* (in the sense that

includes *B. mycoides*). The findings in this investigation and that of Galesloot (6) suggest that in reports where the agents of deterioration are stated to have been active peptonizers of litmus milk, or unnamed spore-formers, or other species of *Bacillus*, the organisms in question are likely to have been *B. cereus* in the great majority of cases.

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## THE INFLUENCE OF OXYGEN ON THE REDUCTION OF NITRATE BY ADAPTED CELLS OF *PSEUDOMONAS DENITRIFICANS*<sup>1</sup>

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### Abstract

The reduction of nitrate by adapted cells of *Pseudomonas denitrificans* has been studied in relation to oxygen concentrations present in the surrounding fluid. No nitrate reduction occurred when oxygen was continually present even at very low concentrations. It appears that the competition between oxygen and nitrate for the donor electrons so favors the oxygen that nitrate reduction only occurs when the supply of oxygen is inadequate to meet the demand.

### Introduction

Skerman, Lack, and Millis (6) demonstrated that, with cultures of *Pseudomonas denitrificans* growing in a yeast extract peptone medium containing nitrate, no decomposition of the nitrate occurred while there was any detectable oxygen in the solution. The results did not indicate whether this was due to inhibition of nitratase formation or inhibition of the action of the enzyme once formed. A critical assessment of either of these questions can only be obtained if they are studied under conditions in which the oxygen concentration is measured in the solution itself and not assumed on the basis of equilibrium with the gas phase. Previous reports of Meiklejohn (3), Sacks and Barker (5), and Marshall *et al.* (2) have unfortunately been based on the latter assumption.

A study has been made of the influence of oxygen on the activity of preformed nitratase in which the oxygen concentration in the solution has been determined and related to the degree of nitrate reduction. The method employed for oxygen determination is essentially similar to that employed in the previous study.

Analyses of nitrate and nitrite were originally made by colorimetric methods. More recently Rand and Heukelekian (4) described a polarographic method employing zirconyl chloride as a supporting electrolyte. Because of the relative rapidity with which these measurements could be carried out, the method was examined and finally adopted in this study.

The materials and methods are given in two parts. Part I deals with the polarographic determination of nitrate. Part II describes the apparatus and procedures used for the anaerobic and aeration experiments.

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## Materials and Methods

### PART I

#### *Polarographic Determination of Nitrate and Nitrite*

Rand and Heukelekian (4) reported that both nitrate and nitrite gave fairly well-defined waves in acid solutions of zirconyl chloride in the absence of oxygen. Practical limits of concentration of the zirconyl chloride were 0.01 *N* to 0.55 *N*. A final concentration of 0.1 *N* was employed in analysis. Nitrate concentrations from 1 to 25 p.p.m. bore a linear relationship to the difference between the diffusion current (*I<sub>d</sub>*) for each concentration of nitrate and the residual current (*I<sub>r</sub>*) obtained after the addition of ferrous sulphate.

Since the buffer-substrate-cells mixtures commonly employed in metabolic studies differ markedly both qualitatively and quantitatively from water and sewage effluents for which the above method was investigated, observations were made on the influence which various substances employed had on the formation and magnitude of the nitrate wave.

##### *(a) The Effect of Phosphate Concentration*

Ten milliliter samples of various phosphate buffer concentrations were mixed with 1 ml. of 1.1 *N* zirconyl chloride and deoxygenated. The polarograms were compared with those of similar concentrations containing 10 p.p.m.  $\text{NO}_3^-$  per milliliter. In *M*/15 phosphate the nitrate wave was completely suppressed. The final pH was 2.75. The absence of the wave was due to precipitation of the zirconyl salt since *M*/15 phosphate is almost twice the final normality of the zirconyl chloride.

In *M*/33 phosphate, nitrate yielded a moderately well-defined wave (Fig. 1). The final solution on dilution is 0.072 *N*, insufficient to cause complete removal of the zirconyl chloride.

In the major portion of our study *M*/15 and *M*/40 phosphate buffer were employed. For nitrate analysis the buffers were diluted to reduce the nitrate concentrations to a measurable range, giving final phosphate concentrations of *M*/950 and *M*/1250 respectively. In such dilutions the nitrate wave (Fig. 3A) is clearly defined and no significant alteration in the pH of the zirconyl chloride buffer is observed.

##### *(b) The Use of Ferrous Sulphate to Obtain a Residual Current Curve*

Rand and Heukelekian (4) used ferrous sulphate to "eliminate" the nitrate wave and stated that "ferrous sulphate is known to reduce nitrate to nitrous oxide (NO) in acid solutions. This is presumably the reaction involved but has not been experimentally proved". Since Tokuoka and Ruzicka (8) had reported that the sulphate ion almost completely eliminated the nitrate wave in lanthanum chloride, it seemed likely that the action of ferrous sulphate was due to the sulphate ion and not to reduction by the ferrous ion. Accordingly, 1.0 ml. of 0.5 *M* ferrous chloride was substituted for the ferrous sulphate.

Fig. 2 shows the residual current curve *A* and the nitrate wave *B* obtained with a mixture of zirconyl chloride and ferrous chloride. The ferrous chloride



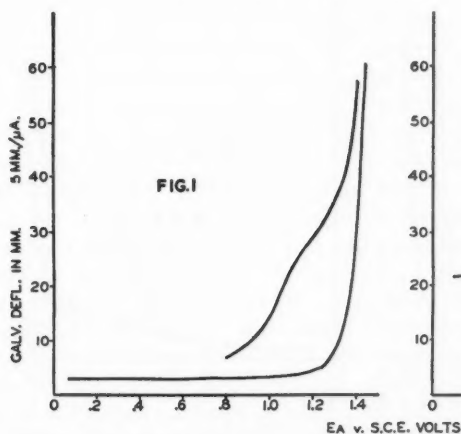


FIG. 1. Polarograms for the residual current and diffusion current for 10 p.p.m. nitrate in  $M/33$  phosphate and  $0.1 N$  zirconyl chloride.

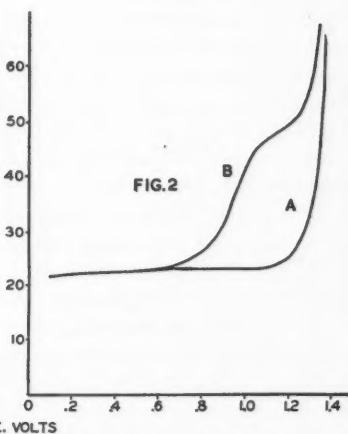


FIG. 2. Polarograms of deoxygenated mixtures.

A. 18 ml. 10 p.p.m. nitrate in  $M/750$  phosphate, 2 ml.  $1.1 N$  zirconyl chloride, 2 ml.  $0.5 M$  ferrous chloride.

B. Similar to A but without nitrate.

causes a slight shift in the decomposition potential of the supporting electrolyte but does not eliminate the nitrate wave. By comparison the addition of ferrous sulphate has entirely eliminated the nitrate wave (Fig. 3B).

Although the ferrous chloride has not eliminated the nitrate wave, Fig. 2A shows that the magnitude of the residual current from 0 to 1.0 volt is considerably larger than that of Fig. 3C. As pointed out by Rand and Heukelekian, this is due to the state of oxidation of the iron. This has been readily verified. With a fresh solution of ferrous chloride the residual current is considerably less. A similar but much smaller rise is seen with the ferrous sulphate (Fig. 3B).

The authors agree with Rand and Heukelekian that provided compensation is made for the change in the residual current on addition of the ferrous salt the magnitude of the nitrate wave remains unaltered. The reduction of nitrate by ferrous iron, if occurring at all, must be exceedingly slow.

### (c) Influence of Substrate

Lactate, acetate, glutamate, aspartate, and yeast extract were used as substrates during the course of the study. Lactate and acetate were used at a concentration of  $M/100$ , yeast extract at  $0.2\%$ , and aspartate and glutamate at 1.0 to 1.5 mg./ml. Each of these was diluted  $1/25$  before analysis. At this level they all depressed the nitrate wave by approximately  $12\%$ . A 20-fold increase in yeast extract (Difco) concentration completely eliminated the wave and yielded a curve similar to that obtained with ferrous sulphate (Fig. 3B). A similar increase in glutamate or aspartate depressed the wave

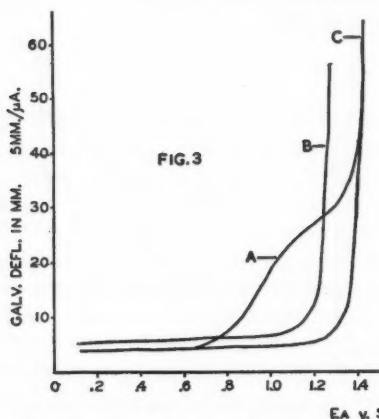


FIG. 3. Polarograms of deoxygenated mixtures.

A. 16 ml. 10 p.p.m. nitrate in *M*/750 phosphate, 2 ml. 1.1 *N* zirconyl chloride, 2 ml. water.

B. 16 ml. 10 p.p.m. nitrate in *M*/750 phosphate, 2 ml. 1.1 *N* zirconyl chloride, 2 ml. 0.5 *M* ferrous sulphate.

C. Similar to A but without nitrate.

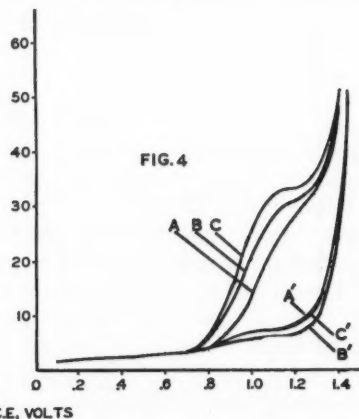


FIG. 4. Polarograms of deoxygenated mixtures.

A. 16 ml. 10 p.p.m. nitrate in *M*/1000 phosphate, 2 ml. *M*/10 lactate, 2 ml. 1.1 *N* zirconyl chloride.

A'. Similar to A but without nitrate.

B. Similar to A but with *M*/100 lactate.

B'. Similar to B but without nitrate.

C. Similar to A but without lactate.

C'. Similar to C but without nitrate.

by ca. 33%. Fig. 4 shows the effect of various concentrations of lactate. Further indication of these effects is seen in the regression lines (Fig. 5) (*vide infra*).

#### (d) Bacterial Cells

Since the concentration of bacterial cells is held constant throughout the experiments, effects of change in concentrations were not tested. However, formalin inactivated cells were always included in the final mixtures used for calibration of the nitrate concentration against diffusion current with the various substrates so that any effect was allowed for in analysis.

#### (e) Urea

It was found necessary to remove nitrite during the course of some analyses by mixing the sample with urea in acid solution. Where this was done, urea was included in mixtures used to obtain the nitrate calibration curve. Its effect was thus eliminated. There was no indication that the maximum concentration of urea employed had any effect on the residual currents.

#### Determination of Proportionality of Nitrate Concentration to the Magnitude of the Diffusion Current Under Different Conditions

Because of the marked positive shift of the residual current obtained when ferrous sulphate was added to the phosphate-zirconyl chloride mixture (Fig. 3B) the procedure recommended by Rand and Heukelekian was

abandoned and a return was made to the more conventional methods of determining diffusion currents. The authors experienced the same difficulty as Rand and Heukelekian (4) in achieving stability in the residual current portion of the curves at potentials more positive than the decomposition potential of the nitrate ion. Curves were found to be more stable in the presence than in the absence of ferrous chloride and the latter was included in all analyses until it was found that ferrous chloride materially affected the nitrite wave (*vide infra*) and had to be discarded in favor of urea. Whilst ferrous chloride was used it was necessary to compensate for changes in the residual current caused by the ferric ion. Rand and Heukelekian did this by "observing its magnitude at the beginning of each day's work and adding the necessary correction to all current changes subsequently observed". This procedure, whilst quite valid, is dependent on uniform deoxygenation of each sample both before and after the addition of ferrous sulphate. Extensive experience with methods of gassing on establishment of equilibrium between the gas and liquid has shown that 5 minutes is not adequate for complete deoxygenation even by methods much more efficient than that implied by Rand and Heukelekian. The second reading taken after regassing following the addition of the ferrous sulphate will usually be lower than the first. For this reason the authors adopted the practice of taking current readings at two potentials (0.5 and 1.2 v.) for both the residual and test waves, after each had been gassed for identical periods. Subtraction of the difference of the former from the difference of the latter gives the true diffusion current and automatically eliminates changes in the residual current caused by the ferric ion and standardizes the effect of any residual oxygen.

The proportionality between the diffusion current and nitrate concentration was plotted under several conditions. The regression lines obtained are shown in Fig. 5. All statistical data relating to these curves are given in the appendix. A linear relationship exists between the nitrate concentration and diffusion current over the range of 0 to 20 p.p.m.  $\text{NO}_3^-$ . Concentrations beyond this range were not tested. It will be noted that the different mixtures affect both the residual current and the slope of the regression lines.

#### *Determination of Nitrite*

Since nitrite is a recognized reduction product of nitrate, the effect of nitrite on the determination of nitrate and methods for removing it had to be examined. Nitrite appeared regularly in anaerobic experiments when cells harvested from a peptone acetate medium were employed with acetate or yeast extract as a substrate. It was rarely detected when cells from a peptone yeast extract medium were used with yeast extract, aspartate, or glutamate as a substrate despite the fact that the pH of the solution was always between 7.0 and 7.5.

Rand and Heukelekian indicated that nitrite yielded a wave in the same position as nitrate but only *ca.* 75% of the magnitude for the same ionic concentration. They did not determine the proportionality.

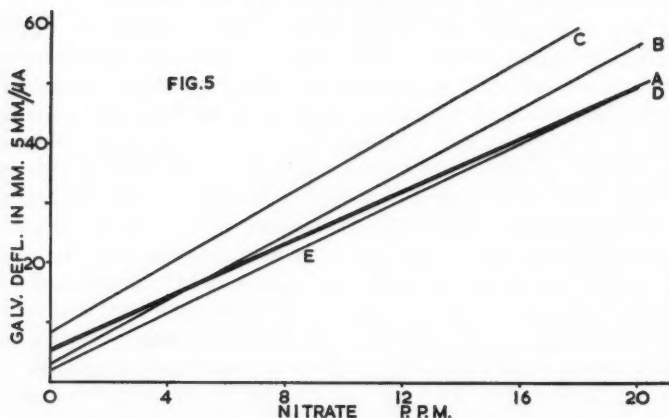
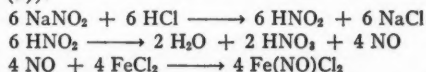


FIG. 5. Regression lines for different concentrations of nitrate in the following mixtures.  
 A.  $M/625$  phosphate,  $0.1 N$  zirconyl chloride,  $0.08 N$  HCl,  $M/1250$  acetate,  $0.08\%$  urea, and  $16 \times 10^7$  bacterial cells/ml.  
 B.  $M/750$  phosphate,  $0.1 N$  zirconyl chloride,  $0.045 M$  ferrous chloride.  
 C.  $M/1250$  phosphate,  $0.1 N$  zirconyl chloride,  $0.08 N$  HCl,  $0.008\%$  yeast extract,  $0.08\%$  urea.  
 D.  $M/1250$  phosphate,  $0.1 N$  zirconyl chloride,  $0.003 M$  aspartate,  $0.08\%$  urea, and  $16 \times 10^7$  bacterial cells/ml.  
 E.  $M/750$  phosphate,  $0.1 N$  zirconyl chloride,  $0.045 M$  ferrous chloride,  $M/500$  lactate.

The production of the wave has been verified. Attempts to determine the linear relationship between concentration and diffusion current have so far been unsuccessful. Whilst a linear relationship can usually be obtained in any one series of titrations, determinations made with fresh solutions on different days continued to yield different results whereas for nitrate the reproducibility under similar conditions is good.

The nitrite wave was not eliminated by ferrous chloride. There was, however, a reduction in the magnitude of the nitrite wave which approximated  $\frac{2}{3}$  of the diffusion current. This may possibly be accounted for by the following equations (Vogel (9)).



(Chloride has been substituted for the sulphate in the original equations.)

From this



If this applies to the reaction, the difference observed would differ from  $\frac{2}{3}$  by the factor representing the ratio of nitrate to the nitrite wave heights for equal ionic concentrations. This is in the order of 5:4. The test has been applied to a few nitrate/nitrite mixtures and found to hold reasonably well for mixtures which are high in nitrite and low in nitrate but not vice versa.

The method may merit further examination from a purely chemical point of view since it would provide a simple method for polarographic determination of nitrate-nitrite mixtures.

### Removal of Nitrite with Urea

Urea reacts readily with nitrite in acid solution yielding nitrogen according to the following equation:



One milliliter of a 2% solution of urea was used for every 1 ml. of sample in which the nitrite ion concentration was limited to 500 p.p.m. and was usually only a fraction of this.

To determine the most suitable procedure for the use of urea the following experiment was conducted.

A mixture "A" consisting of 40 ml. *M*/40 phosphate buffer, 5 ml. *M*/10 sodium acetate, and 5 ml. of 2000 p.p.m.  $\text{NO}_2^-$  was prepared. A control "B" without nitrite was included.

One milliliter of each was added separately to mixtures of 2 ml. *N* HCl, 18 ml. distilled water, and 2.5 ml. 1.1 *N* zirconyl chloride in 25 ml. standard flasks, and each made up to volume. Aliquots were transferred to polarographic cells and deoxygenated with nitrogen at *ca.* 100 ml. per minute for 5 minutes. Readings were taken at 0.5 and 1.2 v. on each sample and then the current was plotted at minute intervals for 10 minutes at the applied potential of 1.2 v. (Fig. 6, A and B). There was no apparent chemical decomposition of the nitrite in the acid solution during this period.

One milliliter of "A" was then added to a mixture of 2 ml. *N* HCl, 18.5 ml.  $\text{H}_2\text{O}$ , and 2.5 ml. 1.1 *N* zirconyl chloride and was deoxygenated, and readings were taken at 0.5 and 1.2 v. One milliliter of 2% urea (previously deoxygenated) was then added and the rate of current change plotted for 10 minutes (Fig. 6C). The decomposition of nitrite was relatively slow.

An attempt was then made to accelerate the reaction by concentrating the main reactants, use being made of the previous observation to measure the change. A series of flasks were prepared each containing 1 ml. 2% urea and

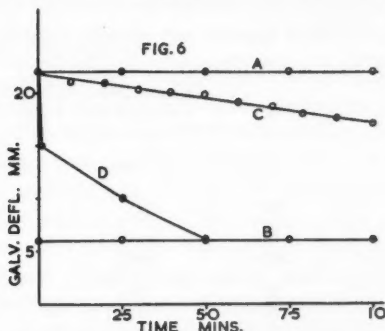


FIG. 6. A. Rate of change in diffusion current for 10 p.p.m. nitrite in a deoxygenated solution of 0.1 *N* zirconyl chloride and 0.08 *N* HCl at 1.2 volt v. S.C.E.

B. Rate of change in the residual current of a deoxygenated solution of 0.1 *N* zirconyl chloride and 0.08 *N* HCl at 1.2 volt v. S.C.E.

C. Rate of change in diffusion current for 10 p.p.m. nitrite in a deoxygenated solution of 0.1 *N* zirconyl chloride, 0.08 *N* HCl, and 0.08% urea at 1.2 volt v. S.C.E.

D. Refer to text.

2 ml. *N* HCl. One milliliter of solution "A" was added to each and one immediately diluted with 18 ml. of water. The others were similarly diluted at intervals of 2.5, 5.0, 7.5, and 10.0 minutes. Zirconyl chloride was added to each and the samples diluted to volume, deoxygenated, and the current measured at 1.2 v. The procedure gives only an approximate estimate of rate since nitrite in the diluted samples continues to decompose slowly during the deoxygenating period. Results are shown in Fig. 6D. Complete removal of nitrite was achieved in 5 minutes and possibly less.

*Determination of Nitrate in the Presence of Nitrite in Experimental Mixtures*

As a final test of the procedure outlined above several experimental mixtures were prepared using in each 40 ml. *M*/40 phosphate buffer in which was suspended inactivated cells of *Pseudomonas denitrificans* to the density employed in the experiments, 5 ml. of substrate (*M*/10 sodium acetate), and 5 ml. of various concentrations of nitrate and nitrite. At the same time a fresh calibration curve for nitrate was plotted in the presence of the cells and substrate. Analyses of the mixtures were made with and without the addition of urea. Data are given in Table I. All readings are given as differences of current readings at 1.2 and 0.5 volts v. S.C.E.

Agreement between the calibration values (column 5) and those determined by removal of nitrite with urea (column 3) is good. An examination of the nitrite figures (column 6) obtained by subtracting column 3 from column 4 shows a linear relationship between nitrite concentration and current for nitrite in the presence of the lowest concentration of nitrate (4 p.p.m.) but considerable deviation at higher nitrate values. The cause of this has not yet been investigated. A similar effect has been reported for analyses performed with ferrous chloride (*vide supra*).

TABLE I  
ANALYSIS OF NITRATE AND NITRITE MIXTURES

Nitrate-nitrite mixtures, p.p.m.		Galvanometer deflection in mm. 5 mm./A = 2.3 p.p.m. NO <sub>3</sub> <sup>-</sup>			
NO <sub>3</sub> <sup>-</sup>	NO <sub>2</sub> <sup>-</sup>	Urea	No urea	NO <sub>3</sub> calibration	Difference due to NO <sub>2</sub>
20	4	48 <sup>2</sup>	51	49 <sup>1</sup>	2 <sup>2</sup>
16	4	42 <sup>0</sup>	42 <sup>2</sup>	41 <sup>2</sup>	0 <sup>2</sup>
16	8	42 <sup>1</sup>	49 <sup>2</sup>		7 <sup>1</sup>
8	4	22 <sup>0</sup>	25 <sup>2</sup>	22 <sup>2</sup>	3 <sup>2</sup>
8	8	22 <sup>2</sup>	33 <sup>0</sup>		7 <sup>2</sup>
4	4	14 <sup>2</sup>	17 <sup>2</sup>		3 <sup>0</sup>
4	8	14 <sup>1</sup>	26 <sup>2</sup>	14 <sup>1</sup>	11 <sup>2</sup>
4	16	14 <sup>1</sup>	42 <sup>0</sup>		27 <sup>2</sup>
0	20	6 <sup>2</sup>	42 <sup>0</sup>	6 <sup>0</sup>	36 <sup>0</sup>

NOTE: Indices on figures in the table refer to 0.25 mm. differences (e.g. 17<sup>2</sup> = 17.75). Galvanometer readings cannot be taken at more accurate intervals because of oscillations.



The final procedure adopted for nitrate analysis was as follows. At selected time intervals a 5 ml. sample was withdrawn from the experimental mixture and three separate 1 ml. aliquots pipetted into 25 ml. volumetric flasks containing 1 ml. 2% urea and 2 ml. *N* HCl. Samples were mixed and allowed to stand for 10 minutes, then diluted, 2.5 ml. 1.1 *N* zirconyl chloride added, and the sample made to volume, deoxygenated, and examined.

## PART II

### *Apparatus and Procedures Used in the Determination of the Influence of the Partial Pressure of Oxygen in Solution on the Activity of Nitratase*

When the work was first begun it was decided to grow the culture under essentially anaerobic conditions in the presence of nitrate, harvest and wash the cells, and suspend them in a buffer in the presence of substrate and nitrate. It was hoped, by comparing the activity of cells incubated anaerobically with those under varying degrees of oxygen partial pressure in solution, to obtain definite data on the influence of the latter on nitrate decomposition.

Preliminary experiments determined the density of cells and the concentration of substrate which were necessary to obtain an appreciable decomposition of nitrate in a reasonable time without depleting the substrate to the level where it would become limiting. Lactate and acetate were employed at a final concentration of *M*/100 and yeast extract at 0.2%. In some experiments—using lactate or acetate—a final density of cells equivalent to *ca.*  $2.5 \times 10^8$ /ml. were used and the time period was 1 hour. With yeast extract, in order to avoid any growth during the experiment, the time was reduced to 30 minutes. In most instances a total volume of 50 ml. was employed, composed of 40 ml. cells in *M*/40 phosphate buffer, 5 ml. *M*/10 substrate, and 5 ml. of nitrate solution. The concentration of the latter varied from 2000 to 5000 p.p.m. in different experiments.

#### *(a) The Anaerobic Experiments*

Before these experiments were conducted a mixture was prepared without the substrate and aerated vigorously. The substrate was added and the time taken for the oxygen to be completely removed was determined. With the constant density of cells employed this was usually 2–3 minutes.

Each anaerobic experiment was conducted in duplicate. Forty milliliters of cell suspension were placed in a 50 ml. volumetric flask and 5 ml. of substrate added. It was allowed to stand for 3 minutes to deoxygenate and then 5 ml. of deoxygenated nitrate solution was added and mixed. After 5 minutes, samples were removed for analysis and the flasks incubated in a water bath at 28° C. The second sample was taken at 35 minutes or 65 minutes. The difference between the two analyses was taken as the index of activity of the culture.

To exclude the possibility that the extreme agitation to which cells are subjected in the aeration experiments may affect their ability to decompose nitrate, the decomposition occurring under the conditions described above

were compared with that occurring under gassing with nitrogen. Commercial  $N_2$ , which contains a small percentage of oxygen, gassing at the rate of 100 to 200 ml./minute through a No. 3 porosity sintered glass filter, never yielded the same extent of decomposition as occurred in the stationary mixtures. Attempts to remove the oxygen by absorption in a chromous chloride column or over heated copper were not completely successful. However, when pure nitrogen was eventually obtained, it was possible to show that the agitation exerted no deleterious effect and that breakdown occurred to the same extent under these conditions as it did in the stationary mixtures.

This method was later substituted for the stationary procedure as a basis for comparison with gas mixtures containing oxygen.

The relationship between nitrate reduction and time under anaerobic conditions has been plotted and shown to be approximately linear.

(b) *The Aeration Experiments*

Foaming of the aerated solutions presented a serious technical problem. Attempts to break the foam with capryl alcohol caused a complete inactivation of the cells and even traces of the material left on the glassware following normal rinsing proved deleterious. Some success was obtained with octadecanol in lard oil. It depressed the foam but had to be continually added during the course of the experiment and frequently resulted in the removal of a large number of the cells from the suspension which were deposited in a scum on the walls of the vessel rendering any accurate comparisons impossible. Resort was made to the use of a sparger to break the foam as it formed. The general design of the apparatus is shown in Fig. 7. Mixtures

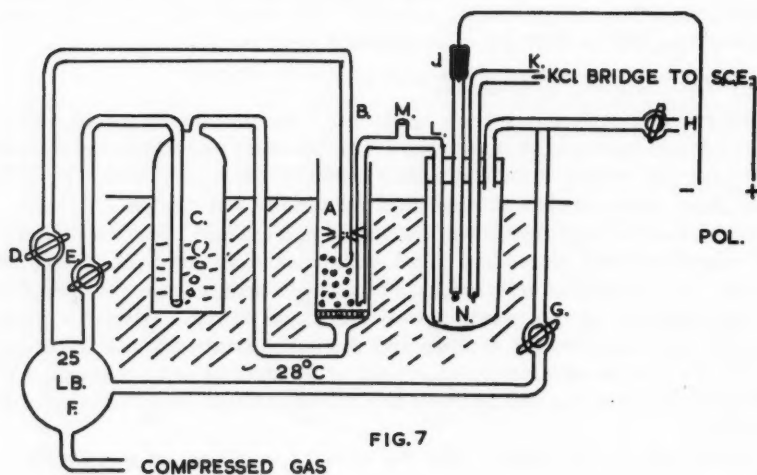


FIG. 7. Apparatus for aeration experiments. See text for description.

of nitrogen and air were prepared in steel cylinders. This mixture was fed through a reduction valve to the manifold *F* whence it could be distributed (a) through valve *E* via a flowmeter (not illustrated) and humidifying chamber *C* to the sintered glass aeration cell *A*; (b) through valve *D* through a second flowmeter and humidifier to the sparger *B*; and (c) through the valve *G* to the polarographic cell *N*.

In operation, a slight positive pressure was exerted on *A* by opening valve *E*. Valve *D* was then regulated to give a flow of 1 liter per minute through sparger *B*. The mixture of substrate and cells was then introduced into *A* and the flow rate through *E-C-A* regulated to 100 ml. per minute. At the 5 minute interval, the oxygen concentration in the sample was determined. The procedure adopted was the same as that employed by Skerman, Lack, and Millis (6). Before the sample was drawn into the polarographic cell, the cell was flushed for several minutes with the gas mixture being used by closing *H* and opening *G*. Just prior to sampling, the flow rate through *G* was reduced and *M* closed to let a few bubbles of gas pass down the tube leading into the polarographic cell. *G* was then closed leaving the system filled with the gas mixture. *H* was then opened and the sample drawn across under vacuum. The oxygen concentration and subsequent uptake rate was recorded on the Cambridge recording polarograph. Immediately the uptake rate was registered the sample was returned to the cell by closing *H* and *M* and opening *G*. The solution was then sampled for nitrate analysis. Thirty or 60 minutes later the second sample was removed for nitrate analysis and the oxygen concentration again determined. Finally the sample in the polarographic cell was reoxygenated with oxygen to a level approximately equivalent to that in equilibrium with air and the subsequent rate of oxygen uptake compared with that at the beginning of the experiment. Since preliminary experiments had determined the quantity of substrate required these rates were always the same unless some change in cell density occurred.

From this it was assumed that since the uptake rate and gassing rate were constant, the level of oxygen recorded at the end of the experiment was the minimum occurring at any stage during the experiment and most likely represented the steady state (Wise (10)).

#### *Media for Cultivation of the Organism*

When acetate was used as a substrate, the organism was grown in the peptone acetate "medium A" of Sacks and Barker (5). When yeast extract was employed as a substrate the peptone yeast extract nitrate medium (YEPW) previously described by Skerman, Lack, and Millis (6) was used. Preliminary investigations had shown that nitratase activity reached its maximum at 48 hours in the former and 24 hours in the latter and the cells were harvested at these time intervals, washed twice in *M*/60 phosphate buffer at pH 7.0, and resuspended for use in *M*/40 phosphate buffer at pH 7.0. The cultures were grown at 28° C. in standing cultures. Oxygen determinations showed that oxygen was depleted at the time a visible turbidity was present.

TABLE II  
INFLUENCE OF OXYGEN CONCENTRATION ON NITRATE REDUCTION

Code	Time, min.	Substrate	$\Delta$ (NO <sub>3</sub> <sup>-</sup> )/ml. expressed as $\Delta$ galvanometer deflection in mm.			<i>p</i> O <sub>2</sub> in the gas	Final <i>p</i> O <sub>2</sub> in solution
			Anaerobic	Gassed			
A	60	Acetate	-43 <sup>0</sup>	+ 2 <sup>3</sup> , + 3 <sup>0</sup>	0 <sup>0</sup>	0.21	ca. 0.21
B	60	"	-31 <sup>0</sup> , -31 <sup>0</sup>	+ 6 <sup>3</sup> , + 2 <sup>0</sup>		0.21	
C	60	"	-49 <sup>0</sup> , -50 <sup>0</sup>	+ 3 <sup>2</sup> , + 2 <sup>0</sup>		0.21	
D	60	Yeast extract	-26 <sup>2</sup> , -26 <sup>0</sup>	+ 1 <sup>2</sup> , + 2 <sup>3</sup> , + 0 <sup>2</sup> + 1 <sup>0</sup> , + 2 <sup>2</sup> , + 0 <sup>2</sup>		0.21 0.21	
E	30	Yeast extract	-17 <sup>3</sup>	+ 3 <sup>2</sup> , 0 <sup>0</sup> , + 1		0.21	ca. 0.21
F	30	"	-17 <sup>0</sup>	+ 1 <sup>2</sup> , + 1 <sup>3</sup> , - 1 <sup>0</sup>		0.068	ca. 0.06
G	30	"	-18 <sup>0</sup> , -16 <sup>0</sup>	0 <sup>0</sup> , + 1 <sup>0</sup> , 0 <sup>0</sup>		0.015	ca. 0.0075
H	30	"	-17 <sup>2</sup>	-15 <sup>2</sup> , -16 <sup>2</sup> , -16 <sup>0</sup>		Commer. N <sub>2</sub>	Nil
J	30	"	-17 <sup>0</sup> , -20 <sup>0</sup>	-12 <sup>0</sup> , -13 <sup>1</sup> , -11 <sup>2</sup>		"	"
K	30	"	-18 <sup>0</sup> , -17 <sup>2</sup>	-13 <sup>0</sup> , -13 <sup>0</sup> , -14 <sup>0</sup>		*	"
L	30	"	-17 <sup>0</sup> , -16 <sup>1</sup>	-15 <sup>2</sup> , -16 <sup>0</sup> , 12 <sup>2</sup>		*	"
M	30	Aspartate	-14 <sup>0</sup> , -13 <sup>0</sup>	-14 <sup>0</sup> , -14 <sup>0</sup>		Pure N <sub>2</sub>	"
N	30	"	-11 <sup>3</sup> , -11 <sup>0</sup>	-11 <sup>3</sup> , -12 <sup>0</sup>		"	"

\*In experiments K and L the nitrogen passed through a chromous chloride column was fed through the cell and nitrogen passed through a heated copper tube was fed through the sparger.

NOTE: Indices on figures in the table refer to 0.25 mm. differences (e.g. 17<sup>3</sup> = 17.75). Galvanometer readings cannot be taken at more accurate intervals because of oscillations.

## Results

The data of several experiments are set out in Table II. The polarographic records showing the oxygen concentration in the sterile cell-free buffer-substrate solution in equilibrium with the respective gas phases and also the final oxygen concentration and uptake rates (where directly measurable) for experiments E, F, G, and J are shown in Fig. 8. Oxygen measurements were made on 0.6 v. S.C.E. For the method of interpretation of these curves, the reader is referred to a previous publication (Skerman, Lack, and Millis (6)).

## Discussion

Table II gives the results of 60 individual experiments carried out using the techniques described in this paper. The number which could be performed on each occasion was limited by the nature of the experimental procedure, each "30 minute" experiment taking approximately 60 minutes to complete. Thus the coded series A to N represent independent series performed with freshly harvested cell suspensions.

The result of each individual experiment is given. With the anaerobic experiments, agreement between duplicates was normally within the limits of experimental error. In the gassing experiments, usually done in triplicate, the agreement between replicates is in most instances quite good. Despite precautions taken to standardize the degree of gassing in each experiment, deviations from uniformity such as that seen in L occasionally occurred.

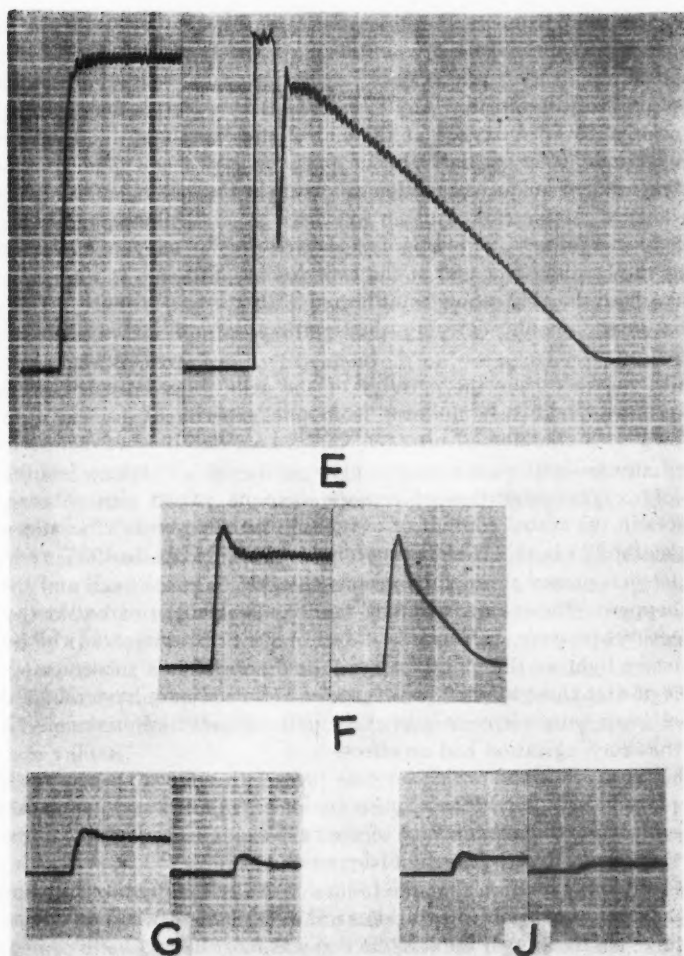


FIG. 8. Polarographic records for experiments E, F, G, and J. See text and Table II.

Apparent increases in nitrate, indicated in A to E where air was used as the gas mixture, sometimes exceeded the allowable error (1.5 mm.) by a significant amount. For this reason no attempt has been made to quote the mean of the readings. This is most likely due to some evaporation, which was inevitable despite precautions taken to humidify the gas mixtures used for the aeration and sparging. The deviation in B is exceptional and no explanation can be offered for it. It is not inconceivable that other factors besides evaporation may be involved.

With all the experiments in which air was employed (A to E) as the gas mixture, there has been either no significant change in nitrate concentration or



a slight increase. In series F and G where the oxygen concentration in the gas phase was reduced to 6.8% and 1.5% respectively, there was still no reduction although there was a quite active anaerobic reduction (equivalent to 200 p.p.m.) of the nitrate. Fig. 8, F and G, show that in both series there was a positive level of oxygen at the end of the experiment equivalent to a  $pO_2$  of 0.06 and 0.0075 respectively.

The first evidence of any reduction of nitrate in the gassing experiments was obtained with commercial nitrogen in which a small percentage of oxygen (0.2–0.5%) was present. Polarograms showed that no oxygen was detectable either at the beginning or end of the experiments although it was detectable in the sterile buffer–substrate equilibrated with the gas mixture. It is clear that the small amount entering the solution did not satisfy the oxygen requirements.

Despite the fact that the activity of the cells judged by the anaerobic breakdown was relatively uniform from one experiment to the next, the breakdown with commercial nitrogen varied considerably between series but was normally uniform within a series of experiments. This suggests that the degree of oxygen saturation of enzyme systems varied either because of differences in the actual content of oxygen in the nitrogen or efficiency of the gassing method or both. The reason for the failure to obtain 100% reduction when nitrogen passed through chromous chloride to gas the cell and through heated copper to the sparger to break the foam was not apparent at the time the experiments were performed. More recent experiments which have thrown some light on these problems will be described in a subsequent paper.

It was at first thought that the degree of agitation may have damaged the cells but when pure nitrogen was eventually obtained commercially it was shown that such agitation had no effect.

Similar experiments in which air was passed through a suspension of the cells in phosphate buffer without substrate for periods of 1 and 2 hours showed that the nitrate reducing capacity of the cells examined anaerobically was in no way impaired either by the agitation or the oxygen.

It was obvious therefore that the failure of the cells to reduce nitrate when the oxygen concentration in solution remained positive was not due to any irreversible inactivation of the enzyme and was most likely due to competition between the oxygen and nitratase for the donor electrons.

The evidence supports the suggestion put forward by the author (6) that inadequate aeration was responsible for the findings of Meiklejohn (3) and Sacks and Barker (5). More recently Marshall *et al.* (2), using isotopic N, claims to have demonstrated that reduction occurs in "aerated cultures" in which "compressed air was passed through sterile filters and into the medium at a rate sufficient to maintain a constant dispersal of minute bubbles throughout. The oxygen tension in the medium certainly approached that normally present in the atmosphere". As it has been repeatedly stated (Finn (1)) that the volume of gas per unit volume of solution is no index of effective oxygenation and that aeration by bubbling is extremely inefficient, it is difficult to see how Marshall *et al.* (2) arrived at the conclusion upon which the ultimate



claims were based. It is extremely unfortunate that such a fine technique as the use of isotopic N was not coupled with an equally sound estimation of the oxygen concentration in the medium. Results obtained in the experiments reported here would suggest that oxygen became a limiting factor in their experiments.

The extremely low levels at which any reduction occurred suggests that nitrate reduction only occurs when the available oxygen fails to satisfy the demand. If this is the case, then the degree of nitrate reduction should decrease as the supply of oxygen increases to the point where the demand is just satisfied. At this stage and not before, the oxygen concentration in solution will show a positive balance. This suggestion was advanced by the author (6) to explain the results of Sacks and Barker (5). It is in close accord with the evidence advanced by Stickland (7) on the relative reaction rates of oxygen and nitrate.

### Appendix

Statistical analysis of data from which the regression lines shown in Fig. 6 have been derived (H. M. Finucan, Department of Mathematics, University of Queensland, Brisbane, Australia).

In the table, values of  $S_y$ , the estimated standard error of  $I_d$  (the diffusion current) at a fixed value of  $N$  (nitrate concentrations) are given. These have been calculated as the sum of squares of deviations from the regression line divided by the degrees of freedom (two less than the number of  $N$  values).

As noted above, three or more observations of  $I_d$  were taken at each value of  $N$ . In most cases, analysis of variance showed the mean square of deviation  $S_y^2$  to be greater than the mean square within arrays (determined from the triplicate values).

Although the "within arrays" mean square varies somewhat from experiment to experiment it is interesting to note that  $S_y$  varies with  $\sigma$ . An estimate of  $S$ , the mean square due to pure deviation from linear regression, is given by  $S^2 = S_y^2 - (\sigma^2/n)$  in the table. The relative constancy of  $S$  will be noted. Repeated determinations (i.e. increase in  $n$ ) or improved techniques (decrease of  $\sigma$ ) could reduce  $S_y$  to the values in the  $S$  column.

Of course the  $S_y$  values are more indicative of precision obtainable with duplicate or triplicate observations.

Code	$\sigma^*$	$n^\dagger$	$\sigma/\sqrt{n}^\ddagger$	$S_y^\S$	$S^\parallel$
A	0.17	3	0.1	0.6	0.59
B	0.62	4	0.31	0.65	0.57
C	1.35	5	0.6	0.8	0.52
D	0.054	3	0.032	0.45	0.45
E	0.53	3	0.31	0.6	0.51

\* $\sigma$  = root mean square deviation within arrays, i.e. of values from the array mean.

$^\dagger n$  = number in each array.

$^\ddagger \sigma/\sqrt{n}$  = "duplicate" or "instrumental" error in an array.

$^\S S_y$  = root mean square deviation of array means from regression line.

$^\parallel S$  = variance ratio.

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## SUSCEPTIBILITY OF MATRIX CONSTITUENTS OF ANTIFOULING PAINTS TO MICROBIAL ATTACK IN SEA WATER<sup>1</sup>

ROBERT L. STARKEY

### Abstract

The rate of loss of the matrix constituents of antifouling paints used on ship bottoms affects the liberation of the toxic substance that prevents fouling. To obtain evidence on the influence of paint composition on antifouling properties of paints, the rate of decomposition and loss in weight of some matrix constituents in sea water was determined. Determinations of each material were made periodically during 6 months. Relative abundance of bacteria in sea water in which the specimens were exposed did not correlate with specimen decomposability. Determinations of oxygen consumption as an index of microbial attack and of weight losses were both similar, and reflected the rate of decomposition. Ester gum and Pentalyn compounds did not decompose. Phenolic resins decomposed slowly, but more rapidly at first than on prolonged exposure. Alkyd resin was relatively resistant but decomposed slowly. Paraffin decomposed slowly at first but rapidly after being kept in sea water for several weeks. Rosins decomposed rapidly from the start. Since the rates of decomposition of some matrix materials were not the same on initial and prolonged exposure in sea water, results obtained during short test periods are not dependable criteria of susceptibility of the materials to microbial attack.

### Introduction

Serviceability of ship-bottom paints in sea water depends on the protection they afford the coated surface against corrosion or other destructive factors, on their persistence, and on their resistance to fouling. The resistance to fouling is due to slow but continuous breakdown of the paint at a rate sufficient to release an amount of the toxic agent lethal to the fouling organisms but slow enough to provide the longest possible period of antifouling action. The minimum adequate leaching rate of copper from paints containing both copper oxide and metallic copper was reported by Ketchum *et al.* (1, 5) to be about  $10 \mu\text{g./cm.}^2/\text{day}$ .

Among the principal paint matrix constituents are rosin and modified rosins, the properties of which greatly affect paint performance. Different matrix constituents such as rosin, modified rosins, synthetic resins, and paraffin are released from paints at different rates with comparable effects on the amounts of the antifouling agent released. The three principal factors responsible for loss of matrix constituents from paints on ship bottoms in sea water are dissolution, microbial decomposition, and mechanical erosion. It was concluded by Ferry and Ketchum (1) that dissolution was the most important factor affecting steady-state leaching.

Most of the information on the influence of physical, chemical, and biological factors on the disintegration of ship-bottom paints is contained in reports to the Bureau of Ships and consequently is relatively unavailable. Many of the

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references are listed in a recent volume on Marine Fouling and Its Prevention (1), in which the importance of the factors affecting fouling are discussed. Darsie (3) reported that rosin dissolved relatively rapidly in sea water in absence of bacteria, whereas Renn (8, 9) found that rosin underwent rapid bacterial decomposition in sea water and concluded that bacterial breakdown of rosin was an important factor in the antifouling action of rosin-containing antifouling paints. Although Darsie (3) also observed bacterial development on rosin, bacteria were thought to grow on physically dissolved rosin with little direct effect on its solubility. The fact that the amount of bacterial slime on paint films increased with increase in the amount of rosin in the paints was interpreted by Renn and Johnstone (10) as further evidence that rosin of the paint matrix was attacked by bacteria. They also found that the copper of certain rosin-containing paints was released more rapidly under non-sterile than sterile conditions.

Although physical dissolution is an important factor in loss of rosin, the reverse is true for paraffin, which is attacked by bacteria (3, 10) and is insoluble in sea water. Various bacteria, actinomycetes, and filamentous fungi decompose paraffin in soil (14). According to Novelli and ZoBell (7) anaerobic sulphate-reducing bacteria decompose it. Copper resinate is also attacked by bacteria in sea water (9). The order of decomposability of some matrix constituents in sea water was reported by Whedon (13) to be as follows (the substance most readily decomposed is given first): WW rosin, 4A rosin, 4B rosin, ester gum, modified phenolic resin F7, cumerone indene. Certain modified rosin compounds and phenolic resins are believed to be relatively resistant to microbial attack (1, 6).

Stahl and Pesson (11) found that various paint plasticizers consisting of esters of sebacic and ricinoleic acids varied in susceptibility to attack by *Pseudomonas aeruginosa* and *Aspergillus versicola* in culture media and that some compounds that were decomposed by one culture resisted attack by the other. Furthermore, it has been stated that alkyd resin (6) and rosin (4) are attacked by fungi. However, one cannot conclude therefrom that they are decomposed in sea water.

In view of the need for additional information to establish the relative importance of microbial decomposition and physical leaching on the loss of matrix constituents from antifouling paints in sea water, determinations were made of their relative decomposability and dissolution. An abstract of some of the material has already been published (12).

### Materials and Methods

The following paint matrix materials were tested:\* wood rosin and gum rosin composed principally of abietic acid; ester gum, which is glyceryl triabietate, a glycerol ester of rosin; Pentalyns A, C, G, and X, which are pentaerythritol abietate resins; alkyd resin (General Electric Co. No. 2466),

\*The Pentalyns were provided by the Hercules Powder Company. Most of the other substances were made available by the Bakelite Corporation.

which is a reaction product of a polyhydric alcohol with a polybasic acid such as a phthalic acid; phenolic resins BR 254 and BR 1329 (Bakelite Corp.); and paraffin.

Bacterial counts in the sea water in which the substances were held showed no consistent correlation with decomposition when measurements were made at infrequent intervals over a period of 5 days. Consequently, oxygen consumption was used as the index of microbial development (2).

Films of the paint constituents were prepared on strips of woven glass tape (Owen-Corning Fiberglass) 1.4 cm. wide and 0.053 mm. thick. The tape was cut in 5 cm. lengths, the ends were fused in a flame to prevent raveling, and the strips were dried at 110° C. They were coated on both sides over a length of 4 cm. to provide an exposed surface of approximately 11.2 cm<sup>2</sup>. The weight of the tape was approximately 200 mg., whereas the attached matrix materials weighed from 100 to 700 mg. Therefore, it was possible to determine weight loss after the tests for oxygen consumption had been concluded.

In preparing the specimens on the glass tape, the Pentalyns, ester gum, paraffin, alkyd resin, and rosins were heated until melted and the strips were immersed in the molten material, cooled, and dried. The phenolic resins were dissolved in methyl-ethyl-ketone and the strips were immersed several times to prepare films of the desired thickness, after which they were dried at 55° C., cooled, and weighed. Ester gum, the Pentalyns, and gum rosin produced the thickest films. The prepared strips were strung on nylon cord with glass spacers to keep the specimens separated. The specimens were held completely submerged in unfiltered, vigorously aerated sea water in glass containers. The sea water was changed at 2-week intervals. Ten specimens of each paint constituent were tested. They were all placed in aerated sea water at the same time and, at approximately 3-week intervals, an oxygen absorption test was made with one sample of each material, after which its weight loss was determined. Therefore, the results represent the oxygen consumption by specimens of matrix materials that had been aged in sea water for different lengths of time. This provided values of the rates of decomposition of the materials during prolonged retention in sea water. The oxygen content of the aerated water in which the specimens were held before the tests were made remained between 6.0 and 7.5 p.p.m. for the test period of 198 days, and bacterial numbers remained high:

In making the tests for oxygen consumption, the specimen was placed in a 250 ml. bottle that was then filled with staled filtered sea water that had been saturated with oxygen just before being used. Glass stoppers were tightly fitted to the completely filled bottles, which were incubated submerged in water held at 20° C. The oxygen content of the water was determined at the start and end of the incubation period. In making the oxygen determinations 200 ml. portions of the water were carefully siphoned from the culture bottle into a 200 ml. bottle in order to obtain water free from the specimen. After the reagents had been added and the reactions completed, duplicate 50 ml. samples were titrated.

The following procedure was followed in testing each specimen when it was removed from the continuously aerated sea water. It was rinsed with fresh sea water and placed in the 250 ml. bottle of sea water for the test for oxygen consumption. At the end of the incubation period of 3 days the oxygen content of the water was determined. The specimen was then rinsed in fresh sea water and placed in another 250 ml. bottle filled with staled filtered sea water saturated with oxygen and incubated for a second 3-day period, after which the oxygen content of the second culture bottle of water was determined. On completion of the test the specimen was rinsed in distilled water, dried, and weighed.

### Results

During the period of 198 days over which the experiment extended, some of the matrix materials showed evidence of alteration. There was no appreciable change in the appearance of the following materials: ester gum, Pentalyns A, C, G, and X, alkyd resin, and phenolic resin BR 1329. There was some loss of material and bleaching of phenolic resin BR 254. The color of the paraffin changed and there was evidence of disappearance of material after 86 days. The rosins showed by far the greatest alteration; there were color changes and at the time the experiment was terminated a large portion of the rosins had disappeared.

From the results of the tests for oxygen consumption the ester gum and Pentalyns A, C, G, and X were not attacked by microorganisms (Fig. 1). The relatively high rate of oxygen consumption for Pentalyn G for the first two test periods was not sustained. The other six materials increased bacterial development significantly (Fig. 2). There was relatively little decomposition of phenolic resin BR 1329 after the first two test periods and only moderate attack of phenolic resin BR 254 and alkyd resin. With these three materials the rate of microbial development was highest initially and there was a general trend toward a decreased rate of decomposition with time.

Although, with paraffin, microbial activity was relatively low at the start, it increased considerably at later periods. This suggests that the population of paraffin-decomposing microorganisms increased on prolonged exposure of the material in sea water. The two rosins decomposed more rapidly than any of the others. Decomposition was rapid at the start and the rate was maintained throughout the experiment. In all cases, practically all of the oxygen contained in the water in which the rosin specimens were tested was consumed, consequently the values for oxygen consumption are minimum ones.

Since with some materials (Pentalyn G, alkyd resin, the phenolic resins, and paraffin) the initial and subsequent rates of decomposition were not alike, a single test period of only a few days is unreliable as an index of susceptibility of matrix substances to decomposition.

In general, the weight losses of the matrix constituents followed courses indicated by the rates of oxygen consumption. The weight losses for ester



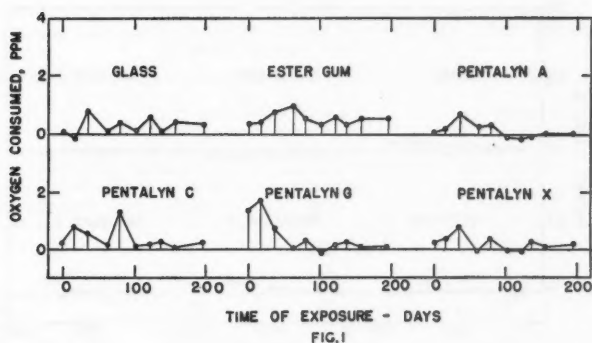


FIG. 1

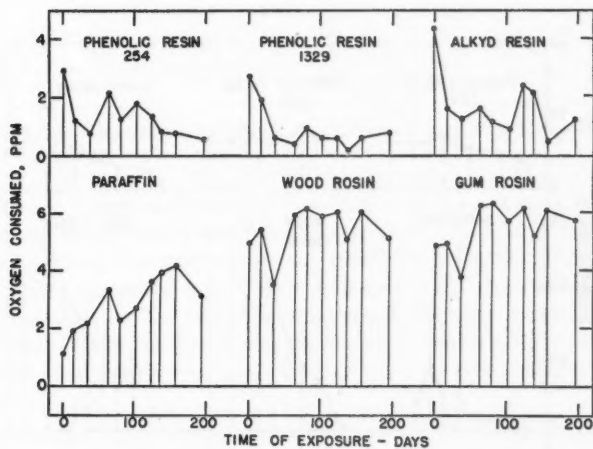


FIG. 2

FIG. 1. Influence of paint matrix constituents, resistant to decomposition, on microbial oxygen consumption in sea water, compared to glass tape control.

FIG. 2. Influence of decomposable paint matrix constituents on microbial oxygen consumption in sea water.

gum and Pentalyns A, C, G, and X were insignificant (Fig. 3). Thus, not only were they resistant to bacterial attack but they were also insoluble in sea water.

Although the initial weight losses of the phenolic resins were high there was relatively little weight loss subsequently (Fig. 4). Alkyd resin was relatively resistant but decomposed slowly. The course of change of the paraffin differed in that there was little weight loss until it had been exposed in sea water for 100 days; the weight losses were 5, 13, 25, and 44% after 86, 107, 142, and 198 days respectively. Both of the rosins decomposed rapidly and at fairly uniform rates. The comparatively small losses in weight at the last two periods are ascribed to reduction in the amount of residual rosin.

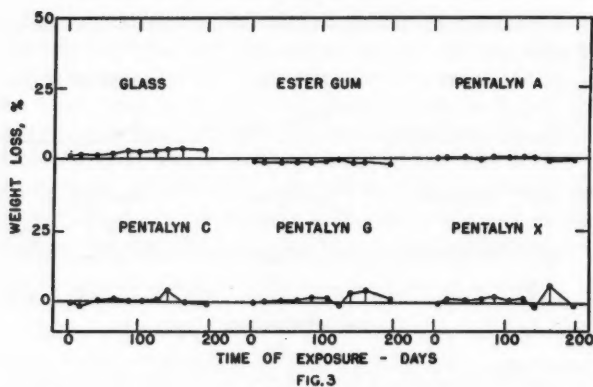


FIG. 3

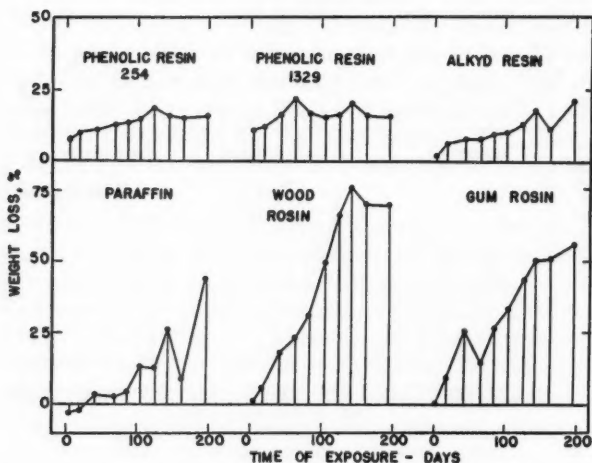


FIG. 4

FIG. 3. Loss in weight of paint matrix constituents, resistant to decomposition, during exposure in sea water, compared to glass tape control.

FIG. 4. Loss in weight of decomposable paint matrix constituents in sea water.

The relative weight losses of the matrix constituents and the values for their effects on oxygen consumption are shown in Table I, with the values for wood rosin being designated as 100. The rosins were the only matrix substances that disappeared continuously and at a relatively rapid rate. Paraffin was fairly rapidly decomposed, but only after several weeks' exposure in sea water. Most of the materials were remarkably resistant to microbial attack and physical leaching.

The paint constituents fall into two groups on the basis of their dissolution and susceptibility to bacterial attack. Ester gum and the Pentalyns were completely resistant to decomposition according to the average results and from those obtained at the last test period. Appreciable amounts of the

TABLE I  
RELATIONS BETWEEN WEIGHT LOSS AND MICROBIAL DEVELOPMENT  
OF PAINT MATRIX CONSTITUENTS\*

Matrix constituent	Last test (192-198 days)		Average of all periods	
	Relative total weight loss for 198 days	Relative amount of oxygen used	Relative weight loss	Relative amount of oxygen used
Wood rosin	100	100	100	100
Gum rosin	79	112	74	102
Paraffin	61	60	23	51
Alkyd resin	30	18	22	27
Phenolic resin BR 254	18	3	28	20
Phenolic resin BR 1329	18	8	36	12
Ester gum	0	3	0	5
Pentalyn A	0	0	0	0
Pentalyn C	0	0	0	2
Pentalyn G	0	0	0	4
Pentalyn X	0	0	0	2

\*In all cases zero values were negative; the weight loss or oxygen used was less than that of the untreated glass tape.

other materials were decomposed. There is relatively good agreement between the values for weight losses and oxygen consumed for all matrix constituents, which suggests that the weight loss was correlated with microbial activity and that the microorganisms were either responsible for the dissolution of the matrix substances, or for their decomposition after the materials had gone into solution. The results indicate that in most cases an estimate of the loss of matrix materials can be obtained by determining either weight or oxygen consumption.

The following is relative decomposability of the matrix materials based on oxygen consumption at the first and last test periods:

#### Initial period

Rapidly decomposed: Wood rosin, gum rosin, alkyd resin  
 Moderately attacked: Phenolic resins BR 254 and BR 1329  
 Slowly decomposed: Pentalyn G, paraffin  
 Not decomposed: Ester gum, Pentalyns A, C, and X

#### Final period

Rapidly decomposed: Wood rosin, gum rosin  
 Moderately attacked: Paraffin  
 Very slowly decomposed: Alkyd resin  
 Not decomposed: Phenolic resins BR 254 and BR 1329, ester gum, Pentalyns A, C, G, and X

Tests have been made also of the decomposability of several other paint constituents but only in test periods of 3 and 5 days. The results indicated that the following materials are very resistant to decomposition: Hercolyn,

chlorinated rubber (Tornesite), chlorinated styrene, coal tar pitch, Halowax, and a vinyl compound VYHH. There was moderate decomposition of both tung oil and linseed oil. The significance of these results is uncertain in view of the fact that the results obtained from tests conducted for short and long periods are not always alike.

### Acknowledgments

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## PREPARATION AND USE OF ENZYMATIC MATERIAL FROM P. LILACINUM TO YIELD CLINICAL DEXTRAN<sup>1</sup>

ARTHUR F. CHARLES AND LEONE N. FARRELL

### Abstract

The development of a method for preparing and standardizing extracellular enzyme from strains of *Penicillium lilacinum* has permitted the degradation of native dextran in such a way that clinical dextran could be separated in reasonable amounts. This enzyme system has been used regularly on crude fermentation broths as well as native dextran. The enzyme system was found to withstand heating at 60° C. for 30 min. within the range pH 6.0 to 8.5. At pH 5.0 activity was almost completely arrested by heating at 60° C. At 23° C. activity was greatest at pH 5.0 to 6.5. It was found to be more labile to acid than to alkali, becoming inactivated at pH 3.0. The optimum medium for enzyme production contained 2% native dextran and 2% corn steep solids. Optimum yield of clinical dextran (50-65% recovery) was obtained when the hydrolysis of the fermentation broth was arrested at  $\eta$ , 2.4. The yield was increased by a second enzymatic hydrolysis of partially degraded dextran which had heretofore not been recoverable for clinical use. The clinical dextran prepared by enzymatic cleavage has complied with the regulations of the Food and Drug Directorate and has been used clinically.

### Introduction

Most preparations of clinical dextran have been obtained from native dextran by acid hydrolysis. It is possible to degrade the macromolecule by supersonic vibration. The classic paper of Wolff *et al.* (8) reviews the literature to 1954.

Dextran degrading substances had been reported by Ingelman in 1948 (3) from *Cellvibrio fulva* and by Hultin and Nordstrom in 1949 (2) from *Penicillium funiculosum*, *Penicillium lilacinum*, and *Verticillium coccorum*. The enzymes produced were low in titer and chiefly intracellular.

We exposed solutions of native dextran to the laboratory air and from those contaminants which produced a decrease in viscosity, indicating dextran degradation, one microorganism was isolated which produced extracellular enzyme with properties useful for our purpose.

This extracellular enzyme appears to differ in several important respects from those reported by Whiteside-Carlson and Carlson isolated from a strain of *Aspergillus* (7) and by Tsuchiya *et al.* in 1952 from various species of *Penicillium* (6).

This report deals with the culture of this organism, the production and standardization of the enzyme system produced, and the use of this enzyme system in the preparation of clinical dextran.

### Experimental

#### Cultures

*Leuconostoc mesenteroides* strain N.R.R.L. B512 was used throughout for production of dextran. It was maintained by weekly transfer in liver extract broth (Haynes (1)). Culture D2 for enzyme production was isolated from a

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Contribution from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario. This work was assisted by a National Health Research Grant.

solution of dextran which was left exposed to the laboratory air. It was identified by Dr. R. F. Cain, Department of Botany, University of Toronto, as *Penicillium lilacinum*. Dr. Cain also supplied two other strains of *P. lilacinum* and one of *Spicaria violacea* for comparative tests. These molds were stored in sterile sand and soil in the refrigerator.

#### *Production of Dextran*

Most of the dextran used was prepared in medium containing 3% corn steep solids, 0.5%  $\text{KH}_2\text{PO}_4$ , and 10% sucrose in 100-liter or 1000-liter stainless steel tanks. Seed culture was built up through tubes of 2% sucrose tryptose broth, 10% sucrose corn steep broth in stirred flasks, and 100-liter tanks for the 1000-liter tanks. Some batches were prepared in yeast extract medium (Rist (4)).

#### *Preparation of Enzyme*

In preliminary experiments, corn steep liquor and yeast extract were compared as sources of nitrogen for preparation of enzyme by strain D2 in culture broths containing native dextran as the carbohydrate. Adjustment of pH was made with sodium hydroxide or potassium phosphate. Corn steep liquor appeared the most satisfactory and was used subsequently. Initial pH of the medium between 6.0 and 8.0 did not significantly affect the yield of enzyme. Sterilization was always at 250° F. for 25 minutes. Erlenmeyer flasks of 500 ml. capacity containing 100 ml. medium were inoculated with spores grown on potato dextrose agar at 23° C. for 8 days. The number of spores was not critical but was usually about 100,000,000. Incubation of flasks was at 23° C. on a rotary shake-table operated at 300 r.p.m. Slower speeds (200 r.p.m.) gave satisfactory results.

#### *Viscosity Determinations*

The relative viscosity ( $\eta_r$ ) of a solution is the ratio of the time required for a measured volume to flow through a capillary compared with the time of flow of the same volume of the solvent. The determinations were made with an Ostwald-Fenske pipette at 25° C. The specific viscosity ( $\eta_{sp}$ ) is equal to  $\eta_r - 1$ . The intrinsic viscosity ( $\eta_i$ ) is the limiting value of the expression  $\eta_{sp}/c$  ( $c$  = concentration of the solute in g. per 100 ml.) where  $c = 0$ . In practice  $\eta_{sp}/c$  is plotted against  $c$  for three concentrations, and the curve is extrapolated to  $c = 0$ . The intercept on the  $\eta_{sp}/c$  ordinate represents the value  $\eta_i$ . When the solute is hydrolyzed dextran, if low concentrations of solute are used (between 0.20 and 0.30 g. per 100 ml.), then  $\eta_{sp}/c$  (viscosity factor) is a close approximation to  $\eta_i$ . Reducing sugars were determined by the method of Somogyi (5).

#### *Clinical Dextran*

Experimentally, we have arbitrarily considered those fractions of hydrolyzed dextran with a viscosity factor between 0.26 and 0.32 to be of a molecular weight within the clinical range. Material for human use has complied with the specifications proposed by the Food and Drug Directorate.



*Effect of pH on Stability of Enzyme to Heat*

Samples of crude enzyme filtrate were adjusted to various reactions from pH 4.5 to 9.0 and heated at 60° C. for 30 minutes. Activity was tested by measuring  $\eta_r$  at appropriate intervals after addition of 0.5 ml. of enzyme to 10 ml. of dextran of initial  $\eta_r$  300. Unheated enzyme reduced  $\eta_r$  to 3.0 in one hour at 24° C. Preparations heated at pH 6.0 to 8.5 were apparently stable. When heated at pH 5.5 the activity was much reduced giving  $\eta_r$  4.5 in one hour, while at pH 5.0 it was almost completely arrested, only reaching  $\eta_r$  240 after one hour.

*Effect of pH on Activity of Enzyme*

The rate of hydrolysis was measured in substrate buffered with citrate over the range pH 3.0 to 4.55 and with phosphate over the range 4.75 to 8.0. Results of this experiment are shown in Fig. 1.

The activity of the enzyme was greatest and essentially independent of pH over the range pH 4.75 to 6.5 declining gradually above, whilst below pH 4.5, there was a sharp decrease in activity to zero at pH 3.0. The action of the enzyme can be arrested by an addition of acid during the course of hydrolysis, as Fig. 2 shows. Below pH 4.0 the action was markedly reduced and was not detectable in one hour at pH 3.35.

The loss of activity shown in Fig. 2 is evidently caused by rapid destruction of the enzyme because enzyme held at 23° C., pH 2.0, for 10 minutes no longer showed any activity when returned to optimum conditions. When treated at 23° C., pH 3.0, for 10 minutes slight activity was detected 16 hr. after restoration to the optimum conditions.

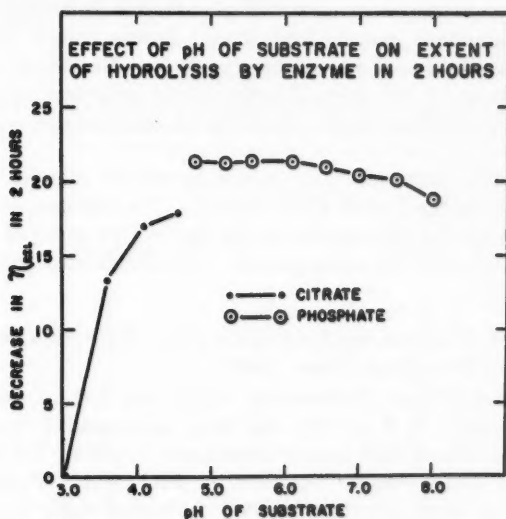


FIG. 1

FIG. 1.

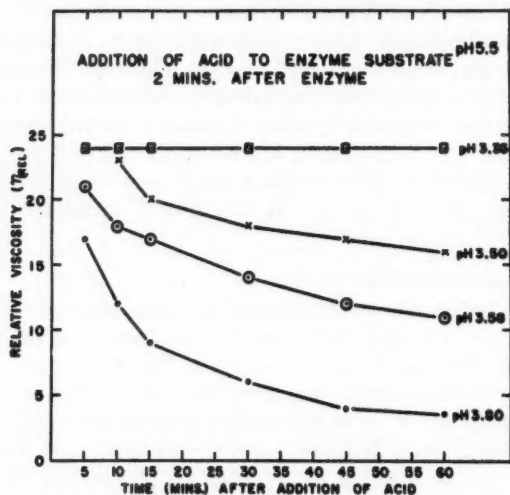


FIG. 2

FIG. 2.

#### *Assay of Enzyme in Terms of a Reference Preparation*

The substrate consisted of 5% native dextran in water mixed with an equal volume of citrate buffer pH 6.0. An enzyme preparation was chosen as a reference standard and arbitrarily assigned a potency of 5 units per ml. To a series of 10 ml. samples of substrate in graduated centrifuge tubes 0.5 ml. of dilutions of standard enzyme from 0 to 1:20 was added, and to another series 0.5 ml. of dilutions of an unknown enzyme. Exactly 15 minutes after addition of enzyme, 1 ml. normal hydrochloric acid was mixed into each tube to adjust the reaction to pH 2.0 and  $\eta_r$  for each mixture was determined at leisure.

Fig. 3 shows that log units of the reference enzyme plotted against log  $\eta_r$  was substantially linear from 0.25 to 5 units. The test sample A referred to this curve gave results also shown in the figure. In practice the standard is used at 0.5, 1.0, and 2.0 units per ml. It is lyophilized and stored in the refrigerator.

#### *Yield of Enzyme Produced in Broth Containing Different Concentrations of Dextran and Corn Steep Liquor Solids*

Replicate flasks (500 ml. Erlenmeyer) containing 100 ml. culture medium at pH 7.6 containing 1, 2, or 3% corn steep solids and 1, 2, or 3% native dextran were inoculated with a spore suspension of culture D2 and incubated at 23° C. on a rotary shaker operated at 250 r.p.m. Flasks containing medium prepared with 1% dextran were harvested daily from the fourth to the seventh day, those with other media daily from the fifth to the ninth day. The contents were centrifuged and the supernatant was filtered through

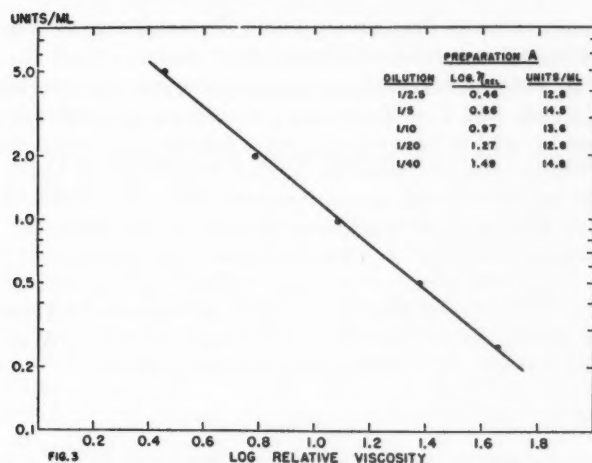


FIG. 3. Assay of preparation A in terms of reference preparation = 5 arbitrary units/ml.

paper and assayed for enzyme by the procedure described above. The results are shown in Table I.

The results in Table I show that for each concentration of dextran used in the culture medium the optimum concentration of corn steep solids was equal weight per cent. The titer of enzyme increased throughout the experimental period, especially in the range of optimum balance of ingredients. For the routine production of enzyme, 2% dextran and 2% corn steep solids was used, with incubation for 8 days for convenience.

TABLE I  
PRODUCTION OF DEXTRANASE (ARBITRARY UNITS/ML.) IN MEDIUM CONTAINING  
DIFFERENT CONCENTRATIONS OF CORN STEEP SOLIDS AND NATIVE DEXTRAN

% dextran	Days after inoculation	% corn steep solids		
		1	2	3
		units/ml.		
1	4	8	7	6
	5	10	8	7
	6	14	9	7
	7	16	10	8
2	5	7	12	19
	6	8	18	22
	7	10	19	22
	8	11	27	26
	9	12	34	24
3	5	5	14	21
	6	6	20	29
	7	5	25	33
	8	8	31	38
	9	11	32	39

*Alcoholic Fractionation of Solutions of Native Dextran Partially Degraded with Enzyme to Different Relative Viscosities*

To 250 g. of 10% solution of native dextran in water, 250 ml. citrate buffer pH 6.0 was added, then 5 ml. dextranase (30 units per ml.) with rapid stirring. The decrease in relative viscosity at room temperature was followed, and samples of 100 ml. removed when  $\eta_r$  reached 3.5, 3.0, 2.5, and 2.0, and at once brought to pH 2.8 with normal hydrochloric acid. Absolute methanol was added to 33% and the precipitate filtered off and discarded. Alcohol was then added gradually to each until the first permanent precipitate appeared. These precipitates were separated and the viscosity factor  $\eta_{sp}/c$  determined. Thereafter, alcohol was added to increase the final concentration in 2% steps, and the precipitates separated at each addition until the viscosity factor fell below 0.25. The results are shown in Table II.

TABLE II

YIELD AND VISCOSITY FACTOR\* OF FRACTIONS OBTAINED FROM 100 ML. SAMPLES OF PARTIAL DEGRADATION OF 5% SOLUTION OF NATIVE DEXTRAN TO RELATIVE VISCOSITY OF 3.5, 3.0, 2.5, AND 2.0

Conc. of methanol (%)	Activity of enzyme arrested at relative viscosity							
	3.5		3.0		2.5		2.0	
	Wt. (g.)	$\eta_{sp}/c$	Wt. (g.)	$\eta_{sp}/c$	Wt. (g.)	$\eta_{sp}/c$	Wt. (g.)	$\eta_{sp}/c$
42	0.39	0.68	0	—	0	—	0	—
43	—	—	0.55	0.47	0	—	0	—
44.5	1.18	0.41	—	—	0	—	0	—
45	—	—	0.80	0.38	0.46	0.37	0	—
46	0.39	0.26	—	—	—	—	—	—
47	—	—	0.55	0.28	0.69	0.30	0.21	0.32
48	0.46	0.24	—	—	—	—	—	—
49	—	—	0.40	0.27	0.53	0.25	0.44	0.30
50	0.37	0.22	—	—	—	—	—	—
51	—	—	0.37	0.34	0.45	0.19	0.46	0.22
53	—	—	0.29	0.23	—	—	0.43	0.20
60	—	—	0.69	0.14	1.34	0.14	1.23	0.13
Total	2.42	>0.24	2.67	>0.24	1.68	>0.24	0.65	>0.24

\*Viscosity factor:  $(\eta_r - 1)/c$  where  $c$  = concentration of dextran of 0.20 to 0.30%.

As may be seen in Table II, the first permanent precipitate obtained from samples removed when  $\eta_r$  had decreased to 3.5, 3.0, 2.5, and 2.0 was at a concentration of 42, 43, 45, and 47% alcohol respectively. When the action of the enzyme was arrested at  $\eta_r$  3.5, nearly 60% of the material precipitated by less than 50% alcohol had a viscosity factor considerably greater than material likely to be of a molecular weight suitable for clinical use. Most of the material from the sample with  $\eta_r$  3.0 had a viscosity factor approaching the desirable range. As the relative viscosity decreased, the yield of material likely to be suitable declined sharply. It was found that when enzymatic action was allowed to proceed until  $\eta_r$  1.38 was reached only 3% of the

glucose moiety of the original dextran was present as reducing sugar. Experiments such as these indicated that this enzymatic material might be used to degrade solutions of precipitated native dextran to material of molecular weight suitable for clinical use.

*Hydrolysis of Native Dextran with Equal Number of Units of Enzyme Provided by Different Preparations*

In a series of experiments, enzyme solutions obtained under conditions other than the optimum as shown in Table I were used to hydrolyze dextran solutions. Enzymes obtained from two other strains of *P. lilacinum* were also used. In each experiment, a volume of 20 ml. of solution containing 80 units of enzyme was used for 1 liter of dextran. Hydrolysis was stopped at  $\eta_r$  2.4 and alcoholic fractionation carried out as above. A control was run with standard enzyme in each test. In every instance, the results of fractionation did not differ from the control by more than experimental variation. The following conclusions are drawn from these tests.

First, the method of assay used gives a true measure of the activity of the enzyme in terms of breakdown of dextran. Second, when hydrolysis is continued to a suitable  $\eta_r$ , the desired products can be obtained. Although selection of colonies from strain D2 led to higher titer of enzyme than was found with the original isolate, or with the other strains tested, strain D2 is not unique in its ability to produce useful enzyme. Attempts to prepare useful enzyme from a strain of *Spicaria violacea* failed, although a dextranase was obtained from it. The enzyme from this mold could not be assayed by the procedure described, and apparently did not degrade dextran in the same way as the filtrates from *P. lilacinum*.

*Hydrolysis of Crude Fermentation Broth with Enzyme to Different Relative Viscosities*

Experiments were done with crude culture broths from *Leuconostoc mesenteroides* fermentation using the enzyme from *P. lilacinum*. Three samples were heated to 80° C., cooled to 23° C. and adjusted to pH 6.0, and the enzymatic hydrolysis was allowed to proceed to  $\eta_r$  3.0, 2.5, and 2.2. After the enzymatic action was arrested, alcohol fractionation was carried out as indicated in Table III.

TABLE III

FRACTIONATION YIELDS WHEN ENZYMATIC HYDROLYSIS OF FERMENTATION BROTH WAS ARRESTED AT VARIOUS RELATIVE VISCOSITIES

% alc.	$\eta_r$ 3.0		$\eta_r$ 2.5		$\eta_r$ 2.2	
	% yield	$\eta_{sp}/c$	% yield	$\eta_{sp}/c$	% yield	$\eta_{sp}/c$
43	29	0.77	23	0.52	9	0.51
47	15	0.41	22	0.36	20	0.36
50	10	0.27	10	0.23	14	0.24

When it appeared from smaller experimental batches that the enzyme hydrolysis produced dextran with the same properties as did the acid hydrolysis, it was necessary that the process be applied to the larger scale operations which had been previously carried out. At the end of the fermentation time the tank and contents were heated to 80° C., then cooled to room temperature (23° C.). The acidity was adjusted to pH 6 and 80 units of enzyme added per liter. The mixture was stirred until the relative viscosity reached 2.4, based on the data of Table III. At this time, acid was added to pH 2.5 and the mixture was warmed to 40° C., resulting in the inactivation of the enzyme. After the acidity had been adjusted to pH 6, the mixture was fractionated with alcohol. The viscosity factor for each of these fractions was determined and the alcohol was added stepwise until a fraction was obtained with a viscosity factor of 0.23 or less. The supernatant from this fractionation was discarded. The fractions with a viscosity factor lying just outside the limits for clinical dextran were refractionated to eliminate the undesirable portions. In general, the yields of clinical dextran produced by enzymatic cleavage were 55–60% of the native dextran in the fermentation broth.

The ease of control of the enzymatic cleavage was demonstrated by the fact that the first fraction, which was too large in molecular weight to be useful as clinical dextran, could be again treated with enzyme to yield about a 20% recovery as clinical dextran. When such a high-molecular-weight fraction was hydrolyzed with acid, the yield of clinical dextran was insignificant.

Sterile non-pyrogenic solutions of dextran prepared in this manner were made available for clinical use, the results of which are being published (9, 10).

#### *Ultracentrifuge Patterns*

The ultracentrifuge patterns of the clinical dextran from both acid and enzymatic hydrolysis are shown in Fig. 4. The materials appear to be alike in homogeneity and sedimentation rates.

#### **Acknowledgments**

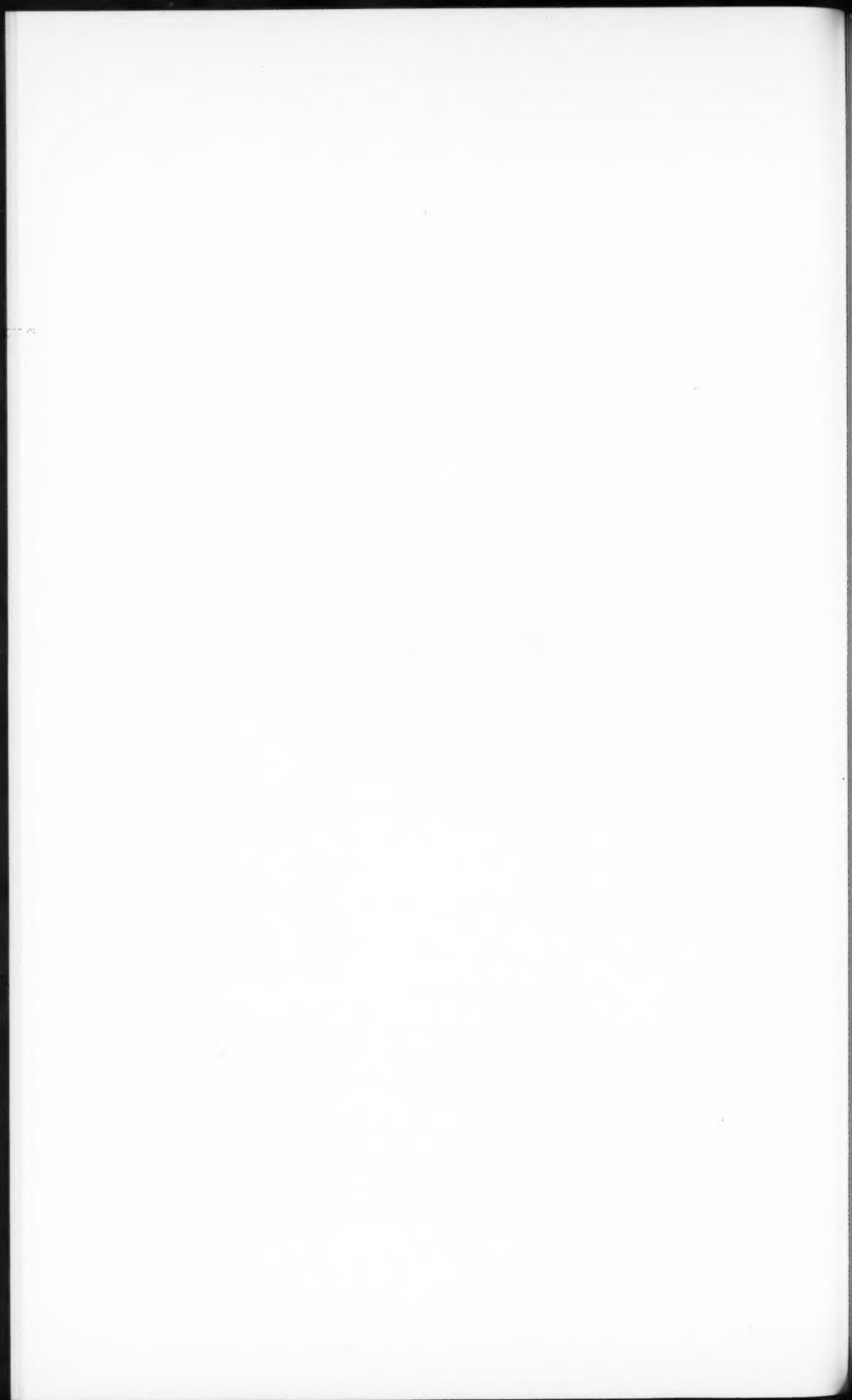
We wish to thank the workers at the Northern Utilization Research Branch, U.S.D.A., Peoria, Illinois, for the culture strain B512, *Leuconostoc mesenteroides*, and for much help in the procedures for production of dextran. Our thanks are due particularly to Mr. C. E. Rist, Dr. A. Jeanes, Dr. F. S. Senti, Dr. W. C. Haynes, and Dr. H. M. Tsuchiya. We are indebted to Professor D. L. Bailey and Dr. R. F. Cain for cultures of *P. lilacinum* and for identification of our own isolate, D2. We also wish to acknowledge the technical assistance of Miss M. Leslie, Mrs. T. Dymont, and Mr. U. Paim. Our thanks are due to Mr. K. A. B. Degen for the ultracentrifuge patterns.



PLATE I

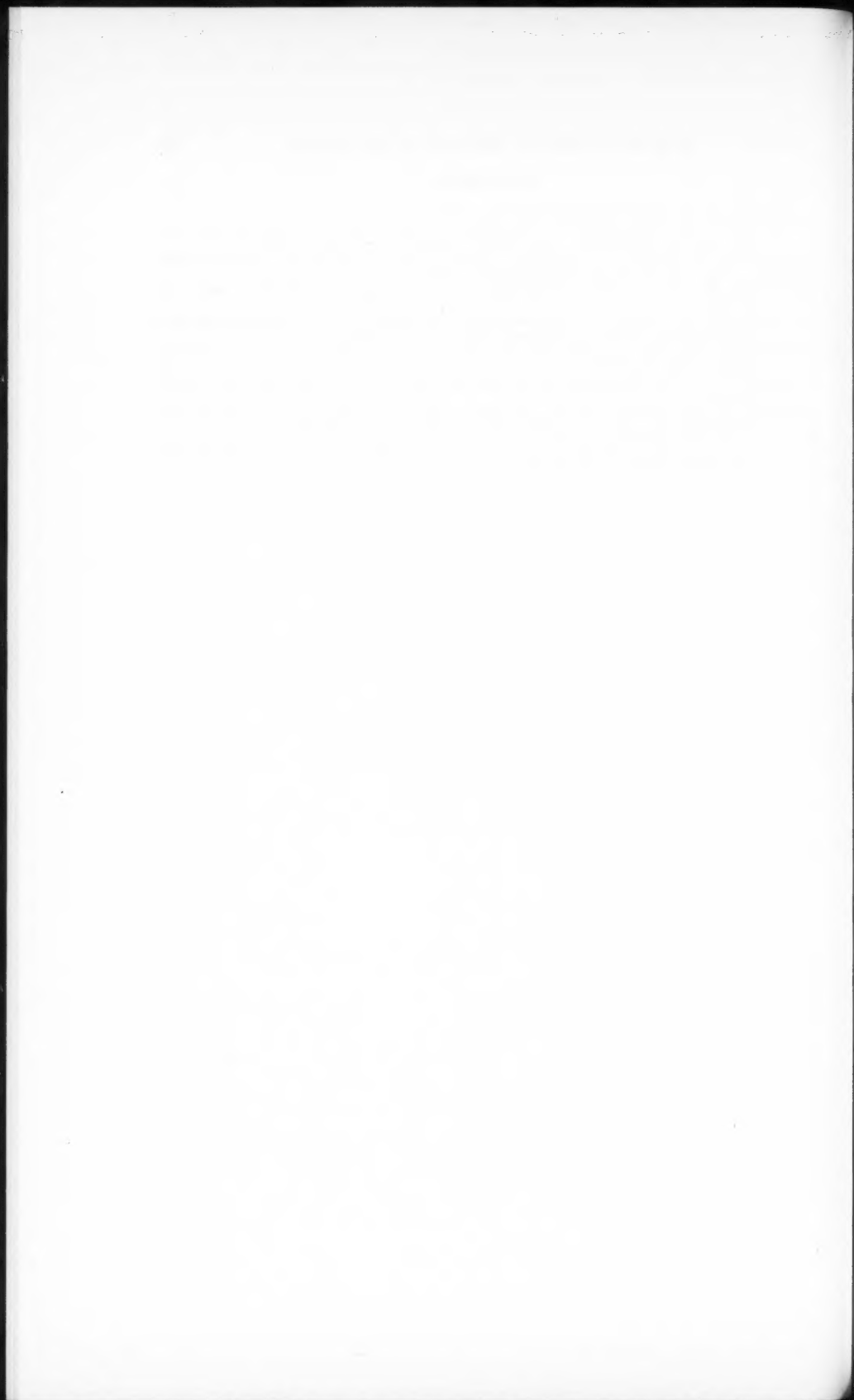


FIG. 4. Ultracentrifuge patterns of clinical dextran from acid and enzymatic hydrolysis. The solutions used were 1% dextran in 1% saline. The length of the run was 40 min. at 59,800 r.p.m. with bar angle 55°. Left=acid hydrolysis. Right=enzymatic hydrolysis.



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## THE EFFECT OF SALT CONCENTRATION ON THE BIOCHEMICAL REACTIONS OF SOME HALOPHILIC BACTERIA<sup>1</sup>

N. E. GIBBONS

### Abstract

Tests with a number of strains of halophilic bacteria indicate that biochemical reactions, such as protein hydrolysis, and indol and hydrogen sulphide production, may vary with the concentration of salt in the medium. It is suggested therefore that, in reporting the biochemical reactions of halophilic bacteria, the concentration of salt in the medium be definitely stated, and that before negative results are reported, the tests be made at different salt concentrations over the range at which the organism grows.

### Introduction

The high salt requirement of halophilic bacteria has hampered the study of their physiology and classification. Although variations in the morphology of the extreme halophiles<sup>2</sup> at different salt concentrations have been reported by many authors, biochemical tests have usually been made in the presence of 20 or 25% salt without regard to the possible effect of salt concentration on these reactions. A few reports have appeared on the effect of salt concentration on the reactions of halotolerant and moderately halophilic bacteria. Garrard and Lochhead (5) found that many organisms, capable of reducing nitrate to nitrite in media containing 5% salt, lost their reducing ability as the salt concentration was increased before they lost their ability to grow. Dumesh (3) noted that decreasing the salt concentration of the medium from 7 to 5% was sufficient to stop the fermentation of glucose and lactose by some strains of colorless halophiles.

The present paper reports the effect of salt concentration on some biochemical reactions of some extreme halophilic bacteria and discusses these effects as they concern classification.

### Methods

Forty-nine strains of extremely halophilic bacteria, including two colorless rods and 47 red-pigmented organisms, were used. The latter were made up of 20 unidentified rods, seven unidentified micrococci, and 20 named species: *Pseudomonas salinaria* (1 strain), *P. cutirubra* (2 strains), *Bacterium (Halobacterium) halobium* (11 strains), *Sarcina littoralis* (1 strain), *Sarcina morrhuae* (3 strains), and *Micrococcus morrhuae* (2 strains). The two colorless

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<sup>2</sup>In this paper, halophilic bacteria are defined as those which do not grow at physiological salt concentrations (0.85%) but require higher concentrations; moderate halophiles, those growing from about 2% to 20%; extreme halophiles, those which require more than 10% salt for growth. Halotolerant organisms are those which are able to grow in the absence of added salt or in the presence of at least 10% sodium chloride.

rods (A2c and A31c) were found as contaminants in two cultures of unidentified red organisms. Six red cultures, five rods and one coccus, were isolated in this laboratory from salted intestines and western Canadian saline soils; the remaining 41 red strains, 29 rods and 12 cocci, including all the named cultures, were obtained from collections in Canada, Norway, Holland, South Africa, and the United States. Many of the cultures isolated by Volcani (11) were obtained from a collection in the United States.

The basal medium contained 10 g. casamino acids (Difco), 5 g. yeast extract (Difco), 2.5 g. L-glutamic acid, 3 g. trisodium citrate, 2 g. potassium chloride, and 25 g. magnesium sulphate heptahydrate per liter. Salt (150, 200, 250, or 300 g.) was added to the above ingredients, and distilled water to a final volume of about 980 ml.; the pH was adjusted to 7.5-7.6 with sodium hydroxide. The solution was then autoclaved at 120° C. for 5 minutes and filtered; the pH was adjusted, if necessary, to 7.0 with hydrochloric acid; 20 g. of agar was added; and the volume made up to 1000 ml. No allowance was made for evaporation during the final sterilization so that the final concentration of salt in the medium was slightly more than 15, 20, 25, and 30 g. per 100 ml. of final medium. Cultures were maintained on this medium containing 20% salt. In later studies, the glutamic acid was omitted and replaced by 5 g. proteose peptone (Difco) and an additional 5 g. of yeast extract. This eliminated the need for neutralizing the acid and the initial heating, so that final concentrations of salt were controlled more accurately. To minimize evaporation and changes in salt concentration, cultures were incubated in a very humid incubator in the first experiments, later in closed jars or screw-capped tubes. All cultures were incubated at 35° C.

Inocula for physiological tests were prepared by suspending the growth from 1-week-old slants in about 3 ml. of sterile 20% sodium chloride solution. A loopful of this suspension was used to inoculate slants or plates and three drops to inoculate broth. Growth at all salt concentrations was usually good to abundant, although occasionally slight growth was obtained at 15 and 30% salt. Five strains of cocci failed to grow at 30% salt in the indol tests and three rods failed to grow in nitrate broth at 15% salt.

Gelatin was sterilized as a 20% aqueous solution and added aseptically to the basal agar to give a final concentration of 0.4%. Hydrolysis was checked after 14 days' incubation by flooding the plates with mercuric chloride solution (8). Soluble starch (0.2%) was added to the basal agar before sterilization and, after 7 days' incubation, hydrolysis determined by flooding the plates with Lugol's iodine solution. For casein hydrolysis, a 20% aqueous solution of Difco skim milk powder was sterilized separately and added aseptically to give a final concentration of 0.8%. Zones of hydrolysis were measured after 7 days' incubation. Hydrolysis of the three fore-mentioned substrates is expressed as the ratio of the diameter of the zone of hydrolysis to that of the colony, so that results are comparable in spite of variations in the amount of growth.



For indol and hydrogen sulphide production, the amount of casamino acids was reduced by half, 0.5% tryptone (Difco) added to the basal agar, and the cultures incubated 14 days. Indol was determined by the Goré method (2). Production of hydrogen sulphide was detected by means of a strip of lead acetate paper inserted in the mouth of the tube. To demonstrate the reduction of nitrate, 0.1% potassium nitrate was added to the basal liquid medium and 10 ml. amounts dispensed into 50 ml. Erlenmeyer flasks. After 4 weeks' incubation, nitrite was determined with Trommsdorf's reagent (4) and nitrate, in the absence of nitrite, with diphenylamine reagent (2).

### Results and Discussion

Two of the main criteria used in the classification of the red halophiles have been their ability to hydrolyze gelatin and casein. In this study, gelatin was hydrolyzed by 45 of the 49 strains tested. However, only a few strains were able to hydrolyze gelatin to the same degree at all four salt concentrations, and the degree of hydrolysis by most strains was influenced by the salt concentration (Table I, A). Some strains showed the greatest gelatinolytic activity at 15% salt with decreasing activity as the salt concentration increased; some strains did not hydrolyze gelatin at 15%, while others did not produce hydrolysis at 30% salt. Fortunately hydrolysis is usually quite marked at 20 and 25% salt, the concentrations used by most workers for biochemical studies. However, in a few instances culturing at only one salt concentration might have led to false conclusions regarding the hydrolytic ability of some strains, e.g. *B. halobium*.

Casein was hydrolyzed by the halophiles much less readily than gelatin (Table I, B). All 22 caseolytic strains were rods and all were also gelatinolytic. The majority of the caseolytic strains hydrolyzed only at the three highest salt concentrations but three strains were hydrolytic only at 20 and 25% salt, one only at 20%, and one only at 15, 20, and 25% salt. Most of the organisms used in this study have been carried on laboratory media for several years and there has been a gradual decrease in proteolytic ability, particularly of casein. For example, our strain of *P. cutirubra* has lost most of its caseolytic ability, although this was one of its distinguishing characteristics when it was isolated by Lochhead (7). Anderson (1) pointed out this loss of proteolytic activity and suggested that classification studies should be carried out only on freshly isolated strains. While this is not always possible, care should certainly be exercised before drawing conclusions from laboratory cultures.

Starch is one of the few carbohydrates attacked by halophiles, and it was hydrolyzed by only nine strains, four rods and five cocci. Whereas all but one (4b1) of these strains were also gelatinolytic, only two (A2c and 4b1) were also caseolytic. Six strains hydrolyzed starch at all salt concentrations and differences were in degree only; two failed to hydrolyze at 30% salt and one at 15% (Table I, C). Starch hydrolysis has been used as a diagnostic reaction in the classification of halophiles but unfortunately some discrepancies

TABLE I  
EFFECT OF SALT CONCENTRATION ON HYDROLYSIS OF GELATIN, CASEIN,  
AND STARCH BY REPRESENTATIVE STRAINS OF HALOPHILIC BACTERIA\*

Representative strain	Salt concentration, %				No. strains showing similar reactions		
	15	20	25	30	Total	Rods	Cocci
(A) Gelatin							
<i>P. cutirubra</i>	10.1†	13.6	12.0	12.2	8	5	3
A 14 (coccus)	3.1	2.9	3.0	3.7			
N2 (rod)	1.3	9.8	8.7	7.0	20	17	3
<i>P. salinaria</i>	0	6.5	9.4	5.8			
<i>M. morrhuae</i> (Delft)	0	5.6	3.6	4.9	11	10	1
<i>B. halobium</i> 6.31.7	0	0	6.4	6.5			
A31	0	3.4	3.9	0	1		1
<i>S. littoralis</i>	2.9	3.4	2.3	0	1		1
A7	11.4	7.9	3.4	2.6	3		3
(B) Casein							
4b2	0	1.6	1.4	1.3	17	17	
<i>H. halobium</i> (Pijper)	0	1.5	1.2	0	3	3	
<i>P. salinaria</i>	0	1.0	0	0	1	1	
A2c	1.8	1.2	1.0	0	1	1	
(C) Starch							
<i>M. morrhuae</i> (Delft)	3.3	5.0	4.3	3.2	6	2	4
<i>S. morrhuae</i> (Volcani)	2.4	4.5	6.1	5.2			
A7 (coccus)	5.8	3.6	3.1	1.0	2	2	1
A2c	3.7	1.8	2.7	0			
4b1	0	1.2	1.6	1.6	1		1

\*In the tables, the actual results obtained with representative strains are presented. These have been chosen to illustrate the different types of reactions to salt concentration without regard to taxonomic groupings of the organisms. The total number of strains having similar reactions to those illustrated is also noted.

†Hydrolysis expressed as the ratio of the diameter of the zone of hydrolysis to the diameter of the colony.

exist in the reported reactions of the various species. Volcani<sup>3</sup> states that *M. morrhuae* does not hydrolyze starch; Venkataraman and Sreenivasan (10) say that it does. Two cultures, received as *M. morrhuae*, originally hydrolyzed starch, although one has now lost this ability, and a strain recently received from Delft does not hydrolyze starch. Since different salt concentrations usually affect only the degree of hydrolysis it is necessary to look elsewhere for an explanation of the variability of starch hydrolysis by cultures of the same species. It is possible that the ability to hydrolyze starch may be lost on long laboratory cultivation, and false conclusions may result. However, this does not offer a complete explanation, since Venkataraman and Sreenivasan (10) state that *Sarcina littoralis* hydrolyzed starch, whereas a strain originally isolated by Lochhead (7) never possessed this ability.

Indol was produced by the majority of strains, only four rods and one coccus giving negative reactions. Although the amount of indol produced

<sup>3</sup>Description of *Micrococcus morrhuae* prepared by B. E. Volcani for the 7th edition of Bergey's Manual of Determinative Bacteriology. The manuscript was kindly supplied by the late Dr. R. S. Breed.

was judged by visual observation only, it was evident that 20 and 25% were the optimum salt concentrations for indol production (Table II, A). The effect of salt concentration on indol production was usually a matter of degree only but indol was not produced at 30% salt by nine strains, at 25 and 30% salt by eight strains, and at 15 and 30% salt by one strain. Of these latter 18 strains, 11 were cocci and include some interesting examples. Our strains of *M. morrhuae* and *S. morrhuae* produced fairly large amounts of indol at 15 and 20% salt but none at 25 and 30%. (*S. morrhuae* sometimes fails to grow at 30% salt.) Volcani<sup>3</sup> reported that *M. morrhuae* does not produce indol. Although he does not give the salt concentration at which indol production was checked, it is presumed to be 24% as used in other tests, and this may account for the negative result reported. It is more difficult to understand the negative results reported by Venkataraman and Sreenivasan (10) for all of the cocci, since presumably they used 20% salt in their media (9).

Hydrogen sulphide was produced by 28 rod forms (Table II, C). The majority of these produced sulphide only at 30% salt (18 strains) or at 25 and 30% salt (nine strains). Only one strain (N3) produced sulphide at 15 and 20% salt and was negative at the higher concentrations. It is possible that the uniformly negative results reported by Venkataraman and Sreenivasan (9, 10) may have been the result of using media containing 20% salt,

TABLE II

EFFECT OF SALT CONCENTRATION ON THE PRODUCTION OF INDOL AND HYDROGEN SULPHIDE AND THE REDUCTION OF NITRATE BY HALOPHILIC BACTERIA\*

Representative strain	Salt concentration, %				No. strains showing similar reactions		
	15	20	25	30	Total	Rods	Cocci
(A) Indol							
5	++	++++	++++	+	19	19	
N2	++++	++++	++++	++			
N5 (rod)	++++	++++	++	+	6	5	1
7	+	++	++++	++	1	1	
A2c (rod)	++++	++++	++	—	9	6	3
A31 (coccus)	++	++++	+	—			
<i>M. morrhuae</i>							
(Dead Sea)	+	++++	—	—	8	1	7
4b1	—	+++	++	—	1		1
(B) Hydrogen sulphide							
N2	—	—	—	+	18	18	
<i>P. salinaria</i>	—	—	+	+	9	9	
<i>B. halobium</i> (M)	—	—	+	+			
N3	+	+	—	—	1	1	
(C) Nitrate reduction							
A2c (rod)	+	+	+	+	5	1	4
<i>S. morrhuae</i> (Volcani)	—	+	+	+	1		1
<i>S. morrhuae</i> (9.5)	—	+	+	—	1		1
<i>S. morrhuae</i> (M)	+	+	—	—	1		1
A31	+	+	+	—	1		1

\*See first footnote to Table I.

although sulphide production has been reported on lead acetate agar containing 20% salt (6, 7). The differences noted here may have been the result of the method used for detection, although the possibility of evaporation in cotton-plugged tubes in other work must also be considered.

Nitrate was not reduced to nitrite by any of the red rods. However, one colorless rod and eight red cocci reduced nitrate to nitrite (Table II, C). Four cocci, including the two strains of *M. morrhuae*, and the colorless rod reduced nitrate at all four salt concentrations. The three strains of *S. morrhuae* reduced nitrate in 20% salt but varied in their response at the other concentrations. The micrococcus, A31, did not reduce nitrate in 30% salt.

In the above studies it soon became evident that the degree of pigmentation often changed with the salt concentration. Usually pigmentation was maximum at a concentration of 20% salt, slightly less at 15%, and decidedly less at 25 and 30%. However, a few organisms became more intensely pigmented as the salt concentration increased from 15 to 30%, while others became less so. There was also a decided difference in the appearance of the colonies of some of the cocci; colonies that were dull and dry in appearance at 15 and 20% salt became smooth and shiny at 25 and 30%.

It should be mentioned that not all biochemical tests are affected by salt concentration, e.g. all organisms tested were uniformly negative for urease and all were uniformly positive for catalase and oxidase at all salt concentrations. However, most of the criteria used for diagnostic purposes are affected to some degree by salt concentration, and it is evident that considerably more knowledge of the biochemistry and physiology of these organisms is needed before their present confused taxonomy can be clarified. Extensive study is necessary before new species are designated on biochemical grounds. It has not yet been determined how the reactions obtained at different salt concentrations are affected by such factors as time, temperature, and pH. It is also not known whether the loss of certain enzyme systems after long cultivation is because of cultivation on unsuitable or incomplete media or whether it is temporary or permanent.

As a result of these studies, it is suggested that, before a negative reaction is reported for any strain of halophile, the test be made at a series of salt concentrations covering the growth range of the organism, and that, for any reaction, definite information be given regarding the composition and salt concentration of the medium and the time and temperature of incubation. Knowledge of the length of time the strain had been maintained in culture and the type of medium used would also be helpful in interpreting data.

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## THE ROLE OF ACID IN POTENTIATING THE ACTIVITY OF PENICILLIN ON LACTIC ACID STREPTOCOCCI<sup>1</sup>

C. K. JOHNS AND I. BERZINS

### Abstract

The inhibitory action of penicillin on cheese 'starter' organisms is potentiated by the acidity developed by these organisms during their growth in milk. If milk containing 0.15 I.U./ml. of penicillin is acidified to *ca.* 0.35% titratable acidity before inoculation, bacterial growth and acid production are almost entirely inhibited, but if the acidity is periodically adjusted to pH 6.4, bacterial growth and acid development are more comparable with those in the control sample. In the presence of 0.15 I.U./ml. penicillin, bacterial plate counts often decreased, while acidity, or turbidity in broth, increased. Direct microscopic counts agreed better with turbidity readings than with plate counts. An apparently greater resistance to penicillin of organisms previously cultured in yeast-phosphate broth proved to be due to the greater number of organisms in the inoculum.

### Introduction

The presence of residual penicillin in milk from cows receiving antibiotic therapy for mastitis has occasionally been responsible for serious difficulties in cheese-making experiments. Acid production by the 'starter' organisms (lactic acid streptococci) proceeded normally during the early stages of cheese-making, but fell off sharply during the 'cooking' process. In several instances the inhibition was so marked that the curd had to be discarded. At first, infection with bacteriophage was suspected, until the presence of penicillin in concentrations in excess of 0.1 units/ml. was definitely established in each case by the disk assay method (2). The studies reported in the present paper were undertaken to clarify the nature of this inhibition, with particular reference to the part played by the acidity developing in milk in which the lactic acid streptococci were growing.

### Review of the Literature

The influence of pH upon the activity of penicillin has been the subject of contradictory reports. With *Staphylococcus aureus*, Foster and Woodruff (5) found that sensitivity to penicillin in nutrient agar at pH 5.5 was more than threefold that at pH 7.0; when the experiment was repeated in liquid media no significant difference at the various pH levels could be detected. Abraham and Duthie (1) reported no difference between the effect of pH 7.5 and 6.5 on the growth of the same organism in broth after 16 hours, and the same was true with the cup assay method. Garrod (7) found penicillin progressively *less* active against *S. aureus* as the pH was lowered from 7.5 to 5.0. Eagle *et al.* (4), on the other hand, reported that the antibacterial

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action against the same organism *increased* as the pH decreased from 7.7 to 5.75; the activity against *Escherichia coli* was affected to a minor degree.

Reports of studies on the effect of pH on the action of penicillin on the lactic acid streptococci have not been found.

### Experimental

Raw milk from the Experimental Farm herd was dispensed in 1000 ml. portions in two 2-liter stoppered Erlenmeyer flasks and heated to 30° C. in a water bath. To one flask was added the desired amount of penicillin;\* the other was left as control. Each flask was then seeded with 2% of a milk culture of *Streptococcus lactis* X which had been incubated at 22° C. for *ca.* 17 hours. After thorough mixing, acidity determinations and plate counts were made immediately and at hourly intervals during incubation. Originally, Hunter's (8) yeast-phosphate-peptone agar was used but marked discrepancies between counts on succeeding dilutions were observed. There was some improvement when tryptone was substituted for peptone in subsequent studies. Incubation was at 32° C. for 2 days.

Data from a typical experiment are shown in Fig. 1. It will be noted that the acidity curve (*p*) for the penicillin-treated milk closely parallels

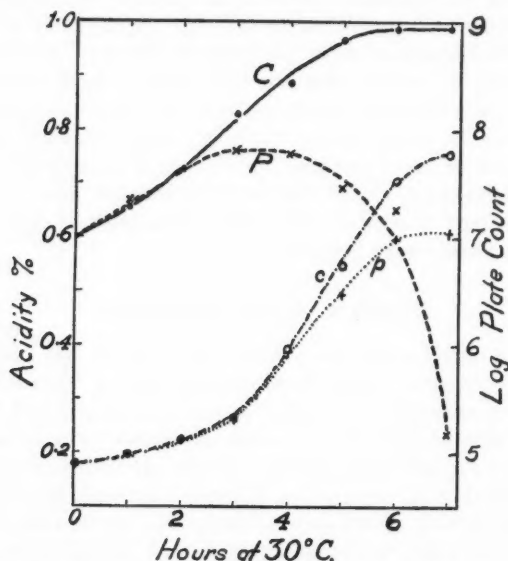


FIG. 1. Bacterial growth and acid development in milk as influenced by penicillin (0.15 I.U./ml.). *S. lactis* X. C = plate count, control; P = plate count, penicillin; c = acidity, control; p = acidity, penicillin.

\*Penicillin-G, manufactured by Eli Lilly & Co. (Canada) Ltd., Toronto. A stock solution was prepared by dissolving 100,000 I.U. in 100 ml. distilled water. This was dispensed in 4 ml. portions and kept frozen until required.

that for the control (*c*) for the first 4 hours, subsequently showing an increasing degree of inhibition. The curve for the plate count (*P*) also showed an increase during the first few hours, after which it declined with increasing steepness, while that for the control (*C*) continued to increase until the 6th hour.

#### Increased Initial Acidity

In order to determine whether the developing acidity was a factor in the stronger inhibitory action of penicillin after the first few hours, several tests were made in which the initial titratable acidity was approximately doubled by bringing the pH to 5.6 with lactic or hydrochloric acid. The results of one such experiment are shown in Fig. 2. It will be seen that with the higher

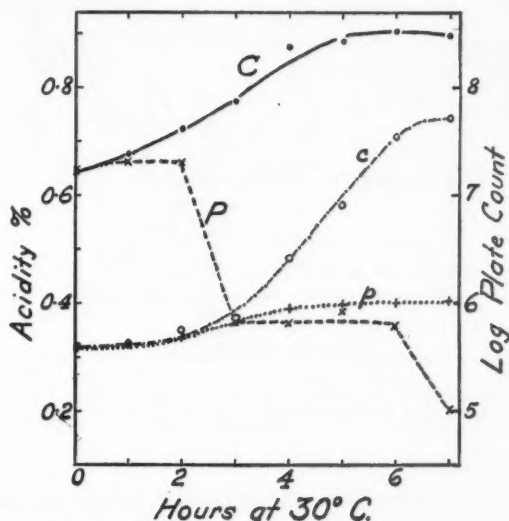


FIG. 2. Effect of increased initial acidity on bacterial growth and acid development by *S. lactis* X in the presence of penicillin (0.15 I.U./ml.).

initial acidity, the 'p' milk showed both earlier and much more severe inhibition of acid production, while the plate count also declined much earlier. The 'c' milk developed a final acidity similar to that of 'c' in Fig. 1. When acetic acid was used to increase the initial acidity, acid development was interfered with in both flasks.

#### Hourly Neutralization of Acidity

In order to obtain further evidence, several tests were made in which both portions of milk were adjusted to pH 6.4 at approximately hourly intervals. The results of such an experiment are presented in Fig. 3. It is evident that this adjustment does interfere with the inhibitory action of penicillin as shown both by acid production and plate counts, but is not sufficient to prevent it entirely.

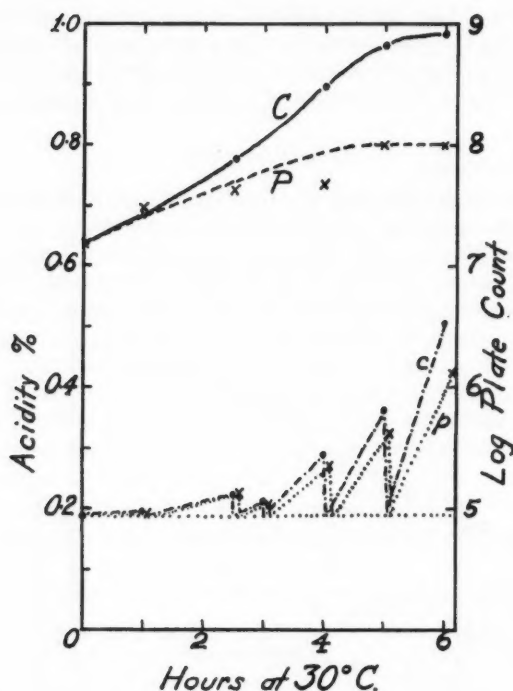


FIG. 3. Effect of periodic adjustment of acidity upon bacterial growth and acid development by *S. lactis* X in the presence of penicillin (0.15 I.U./ml.).

#### Growth in Broth Containing Penicillin

Since penicillin interferes with cell division, it seemed probable that the plate counts obtained from milk containing penicillin might not be a reliable index of the true extent of bacterial growth. Consequently, the increase in turbidity in broth containing various concentrations of penicillin was determined. Yeast-whey broth\* was inoculated with 1% of an overnight milk culture of *S. lactis* X, and incubated overnight at 22° C. Next morning 0.5 ml. of this intermediate culture was added to 10 ml. of yeast-whey broth containing suitable concentrations of penicillin. At intervals during incubation in a water bath at 30° C., the turbidity was measured with a Klett-Summerson photoelectric colorimeter using a green filter, and the colony count determined by the plate method and the individual cell count by the direct microscopic method (2).

The results from one such experiment are shown in Fig. 4. The turbidity values indicate a considerable increase in cell volume despite the drop in plate count after 2 hours in the presence of 0.15 units/ml. penicillin. The

\*Difco whey broth plus 0.25% yeast extract.

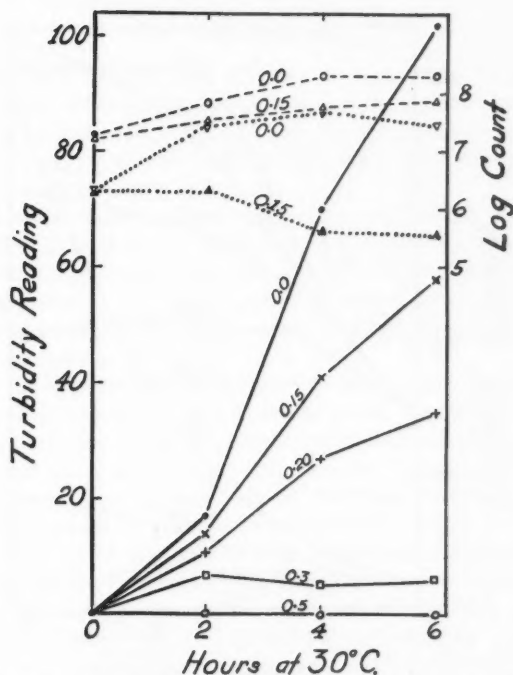


FIG. 4. Effect of penicillin on growth of *S. lactis* X in yeast-whey broth. --- = direct microscopic count of individual cells. .... = plate count. — = turbidity reading.

direct microscopic count also increased in the presence of 0.15 units/ml.; the cells were abnormal and swollen (6) and in much shorter chains than in the control milk.

That milk is a much superior medium for the growth of the lactic acid starter organisms is indicated by the significantly higher plate counts obtained from this medium than from yeast-whey broth (cf. Fig. 4 with Figs. 1-3).

#### Results with Other Lactic Acid Streptococci

In order to determine whether the results reported above were typical for the lactic acid streptococci, similar studies were done with two strains of *S. cremoris* (HP & C13), two mixed culture cheese starters, and one represented to contain several strains of *S. lactis*. Essentially the same results were obtained in every case.

#### Influence of Culture Medium on Resistance to Penicillin

In view of the rather slow growth of *S. lactis* X in yeast-whey broth, some comparisons were made using yeast-phosphate broth\* as the intermediate culture medium for turbidity studies. As is evident from the results in

\*Yeast-phosphate-tryptone agar minus the agar.

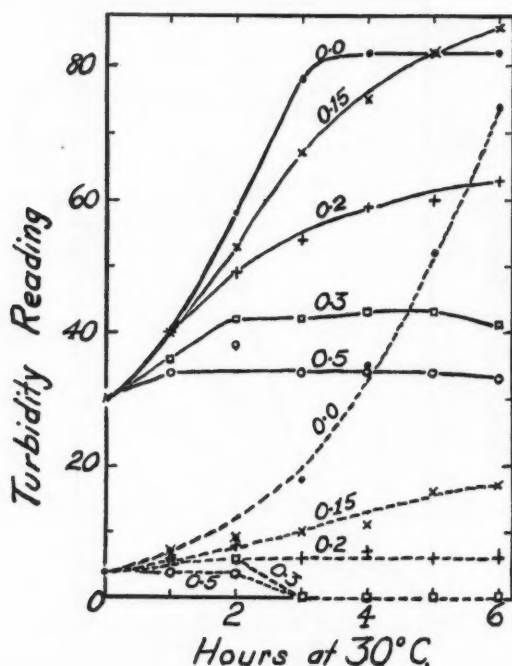


FIG. 5. Growth of *S. cremoris* C13 in yeast- whey broth. Upper series of curves represent cultures inoculated from yeast-phosphate intermediate culture; initial plate count 3,900,000/ml. Lower series inoculated from yeast- whey intermediate culture; initial plate count 1,200,000/ml.

Fig. 5, with *S. cremoris* C13, growth in the broth inoculated with the yeast-phosphate broth culture was much heavier. Even more interesting was the much greater degree of resistance to penicillin of the organisms grown in this medium; a concentration of 0.3 units/ml. was no more inhibitory here than was 0.15 units/ml. when yeast- whey broth was used as the intermediate culture medium.

The seemingly greater resistance to penicillin displayed when the organisms were grown in yeast-phosphate broth prior to the turbidity testing was more apparent than real. This was shown in subsequent studies where the number of organisms present at the start was varied. When the inoculum from the yeast-phosphate broth contained approximately three times as many cells as that from the yeast- whey broth, growth in the presence of penicillin was very much stronger; when both inocula contained approximately the same numbers, there was little difference; when the inoculum from yeast-phosphate broth contained one-third as many cells, growth in the presence of penicillin was much lighter.



### Discussion

The results from studies with *S. lactis* X show that the acid developed by the organisms growing in the milk definitely potentiates the action of penicillin. That this probably holds true for the group of lactic acid streptococci is indicated by similar findings with other representative strains and mixed culture starters.

Where the initial acidity of the milk was approximately doubled (Fig. 2), it was surprising to note a sharp deflection in the curve for the log plate count of the penicillin-treated milk after 2 hours, following which the count levelled off for several hours. The experiment was repeated three more times, and each time the same phenomenon was noted. No explanation for this peculiar effect has so far been found.

Results of studies with staphylococci (3) have indicated that lysis of the cells appears shortly after growth is inhibited by penicillin. This does not appear to hold true for the lactic streptococci. Despite the decrease in plate count, the direct microscopic count and turbidity values increase for at least 6 hours, and in many cultures during overnight incubation. Even 200 I.U./ml. failed to produce lysis of *S. cremoris* C13.

Throughout these studies there was considerable difficulty in getting reasonable agreement between plate counts of different dilutions, and sometimes even on duplicate plates of the same dilution. This difficulty was quite pronounced when yeast-phosphate-peptone agar was used. Although the substitution of tryptone for peptone effected a considerable improvement, poor agreement was still too frequent. This was true with all six starters studied, and was not confined to counts from penicillin-treated milk. However, each test was replicated a sufficient number of times to make certain that the trends were generally as depicted in the graphs.

One difference between these experiments and the actual cheese-making procedure lies in the fact that in cheese-making the milk is curdled by the action of rennet. This could conceivably result in an increase in the concentration of penicillin within the curd particles, where active acid production by the streptococci is vitally important as, for example, in Cheddar cheese-making. Laboratory tests simulating cheese-making procedures showed a slight decline in the concentration of penicillin in a whey sample taken soon after coagulation and cutting of the coagulum; in portions of whey exuding from the curd an hour or two later, the concentration of penicillin showed an increase. This increased concentration of the penicillin in the curd is probably a factor, along with increasing pH, which helps explain why the inhibition of acid development in the cheese vat increases in intensity as the cheese-making process progresses.

### Acknowledgments

The authors are indebted to Dr. H. Katznelson for helpful suggestions and encouragement, and to the New Zealand Dairy Research Institute for kindly furnishing the cultures of *S. cremoris*.

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## STUDIES ON THE INCIDENCE OF CERTAIN PHYSIOLOGICAL GROUPS OF BACTERIA IN THE RHIZOSPHERE<sup>1</sup>

H. KATZNELSON AND J. W. ROUATT

### Abstract

It has been demonstrated by the ultimate dilution method that the rhizospheres of field crops such as wheat, oats, rye, and barley harbor a bacterial population which is physiologically more active than that of non-rhizosphere soil. This was shown by use of the following criteria: reduction of methylene blue or resazurin, production of acid and gas from glucose, and ammonifying and denitrifying capacity. In general a higher proportion of isolates from the rhizospheres of wheat, barley, and rye were capable of reducing methylene blue, producing acid from glucose, and producing ammonia from peptone than from control soil. The significance of these and the above results is discussed.

### Introduction

It is now well established that soil in the immediate vicinity of plant roots supports larger numbers of microorganisms than soil at a distance from the roots. Bacteria, fungi, actinomycetes, and other organisms have been shown to increase at the root soil interface, which has been named the rhizosphere (2). Much less is known, however, of the qualitative nature of the microflora in this zone of activity. This is a more difficult problem because of the lack of a uniform and generally accepted means of characterizing the soil microflora. In recognition of this situation Lochhead and associates (8, 17) devised a scheme for classifying the predominant bacterial flora of the soil on the basis of nutritional requirements. The value of this method is attested by the various significant contributions deriving from its application, among which are: the concept of the bacterial balance index (17); the discovery of the shift in the bacterial equilibrium in rhizosphere soil in favor of bacteria requiring amino acids for optimum growth (9, 10); and the demonstration that many soil bacteria require vitamin B<sub>12</sub> and other known and unknown growth promoting substances (6, 7, 11).

The nutritional approach demonstrated that a qualitative difference exists between the microflora of the soil and of the rhizosphere. This conclusion is supported also by classical bacteriological methods, with which Lochhead showed (5) that there was a greater percentage incidence of Gram-negative rods, of motile and chromogenic types, and of bacteria producing good growth in nutrient agar in rhizosphere than in non-rhizosphere soil. More recent studies have provided even more evidence along these lines. Particularly striking was the preferential effect exerted in the rhizosphere on ammonifying and denitrifying bacteria (1, 3). Fungi, protozoa, aerobic cellulose decomposing, anaerobic gas producing, and anaerobic bacteria were found to be more abundant in the rhizosphere of mangels (1) and wheat (3). On the other hand, no increase in numbers of *Azotobacter* or of nitrifying bacteria was observed in the rhizosphere of wheat, barley, or soybean (3).

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Algae appeared to be inhibited by wheat roots. A selective action in the rhizosphere has been reported also by Rovira, who studied the rate of growth of a small number of soil and rhizosphere cultures isolated at random from agar plates (14) and the relative prevalence of chromogenic and certain morphological types of bacteria (15). King and Wallace (4) also observed a selective effect in the rhizosphere on Gram-negative rods; they found, however, that one physiological group, the nitrate-reducers, was proportionately increased in the rhizosphere soil of young oat plants.

During the past year, emphasis in this laboratory has been placed on the relative growth rates of soil and rhizosphere bacteria and on their physiological behavior as determined by the production of acid and gas from carbohydrates and by dye reduction. The results of the experiments on growth rates are presented in a separate paper in this issue (13) and those dealing with physiological activity are described herein.

### Experimental

Soil and rhizosphere samples were obtained from the field in the usual manner (5) and suitable dilutions were prepared. One milliliter aliquots of each dilution were added in quintuplicate to tubes of appropriate media which were incubated at 26° C. and examined at regular intervals. Studies on bacterial isolates from soil and rhizosphere were carried out by plating samples with soil extract agar and isolating all the colonies on a plate or representative section thereof after an incubation period of 14 days. The colonies were picked and stab cultures made to soil extract semi-solid agar and, after 5 days' incubation, transfers were made to medium S (8), which contains soil extract, inorganic salts, and 0.1% glucose; peptone and yeast extract were also added at the rate of 0.25%. For the dye reduction tests both methylene blue and resazurin were used in a final concentration of 1 : 200,000 in test tubes. Acid and gas production were determined with Durham tubes in the above medium containing bromocresol purple at a final concentration of 0.0008%. Ammonia production and denitrifying ability were determined in suitable media as indicated previously (1).

### Results

The data from the first series of tests are summarized in Table I. The wheat and oat rhizosphere samples used in these experiments were taken from young plants (5 weeks) in the spring, whereas the rye and barley plants were near maturity when sampled. It may be concluded that the rhizosphere harbors a microflora which is indeed more active physiologically than that of the adjacent soil. It is clear also that crop differences occur since under comparable conditions the rhizosphere : control (R : C) ratios of oats and rye are markedly lower than those of wheat and barley.

The cultures isolated from the above samples were inoculated into several differential media to determine the percentage incidence of the physiological types indicated in Table II. It will be noted that a greater proportion of

TABLE I  
INCIDENCE OF CERTAIN PHYSIOLOGICAL GROUPS OF SOIL  
BACTERIA IN RHIZOSPHERE AND CONTROL SOILS\*

Physiological groups	Rhizosphere						Rhizosphere							
	Control	Wheat			Oats			Control	Rye			Barley		
		No.	R:	C†	No.	R:	C		No.	R:	C	No.	R:	C
Reducing methylene blue	12.5	506.0	40	147.0	12	1.3	8.2	6	26.4	20				
Producing acid from glucose	2.5	200.0	80	9.2	3.7	0.34	2.1	6	130.0	382				
Producing gas from glucose	0.2	27.0	135	0.02	0.1	0.003	0.04	13	0.2	66				
Ammonifying	7.7	530.0	69	30.0	3.9	0.98	33.5	34	60.3	62				
Denitrifying	0.04	5.3	132	0.21	5	0.03	0.39	13	1.9	63				

\*Expressed in millions per gram oven-dry soil. Calculations based on most probable numbers.

†R : C =  $\frac{\text{Numbers in rhizosphere soil.}}{\text{Numbers in control soil.}}$

TABLE II  
INFLUENCE OF RHIZOSPHERE ON THE PERCENTAGE DISTRIBUTION OF CERTAIN  
PHYSIOLOGICAL TYPES AMONG REPRESENTATIVE BACTERIAL ISOLATES\*

Physiological types	Control	Rhizosphere		Control	Rhizosphere	
		Wheat	Oats		Rye	Barley
Reducing methylene blue	20	68	23	30	23	82
Producing acid from glucose	2	9	2	3	14	23
Ammonifying	17	40	13	18	40	47
Bacterial count†	64	2124	600	115	1156	2622

\*Approximately 100 isolates were tested in each case.

†Millions per gram oven-dry soil.

the bacteria from the rhizosphere of wheat and barley than from the control soil were capable of reducing methylene blue. A greater proportion of rhizosphere organisms were also capable of fermenting glucose and of producing ammonia. Isolates from oats, however, showed no such differences.

Further tests were carried out with very young oat and barley plants growing in a different field in the fall, after a particularly cool and moist summer. These tests were undertaken to obtain more data on the relative abundance of certain groups of organisms in the rhizosphere and to compare several other dyes with methylene blue as a means of determining the oxidative capacity of the soil microflora. Triphenyl-tetrazolium chloride (TTC) was incorporated into soil extract agar aseptically to give a final concentration of 0.001%. Again a distinct rhizosphere effect may be observed (Table III), not only with methylene blue as indicator but also with resazurin. TTC in the amount used appeared to exert an inhibitory effect on the bacterial count. Nevertheless an increase in number of TTC-reducing colonies occurred in rhizosphere samples. Since the R : C ratios were of the same order as those of the total bacterial count it may be concluded that a selective action on these organisms was not exerted in the root zone.

### Discussion

The observation of Lochhead (5) that "in the rhizosphere the bacteria show definitely greater physiological activity than in soil distant from the plant" is fully supported by the data presented above despite the fact that several quite different criteria were employed as indices of physiological activity. The use of methylene blue or resazurin offers a simple and rapid means of determining the metabolic activity of the soil microflora. On the basis of the available data, however, it is not possible to select one or the other of these dyes as the better for the purpose. Resazurin is not reoxidized as readily as methylene blue and is perhaps more sensitive; on the other hand higher R : C ratios were obtained with methylene blue (Table III).

From the results of Lochhead (5), Rovira (14, 15), Rouatt and Katznelson (13), and those recorded in this paper it would appear that the more active and rapidly growing soil bacteria gain ascendancy in the rhizosphere. This is to be expected in any complex natural environment where an intense struggle for existence is going on. On a macroscopic scale this competition results in the eventual dominance of certain plant communities such as grasses or forest trees. On a microscopic scale it results in the preponderance of those microorganisms which are most active physiologically and which can grow most rapidly, as in the rhizosphere. There remains to be determined the composition of this microflora in order that the predominating types may be ascertained more precisely. This is of importance from a practical as well as an academic point of view as has been amply demonstrated in studies on strawberry root rot (16) and potato scab (12). Information is also needed on the specific effects of the predominating forms in the rhizosphere, on the plant itself. The elucidation of these problems may be of great practical and fundamental significance.

TABLE III

INCIDENCE OF CERTAIN PHYSIOLOGICAL GROUPS OF BACTERIA IN THE RHIZOSPHERE OF SEEDLING OATS AND BARLEY\*

Physiological groups	Control	Rhizosphere			
		Oats		Barley	
		No.	R : C†	No.	R : C
Reducing methylene blue	4.1	700.0	171	4100.0	1000
Reducing resazurin	58.0	8200.0	141	2500.0	43
Producing acid from glucose	1.6	7.0	4	31.0	19
Producing gas from glucose	0.014	3.2	229	0.27	19
Bacterial count	185.0	1275.0	6.9	1490.0	8.0
Count on TTC agar‡	95.0	775.0	8.1	1045.0	11.0
Colonies reducing TTC	27.0	225.0	8.1	224.0	8.0

\*. †See footnotes Table I.

‡Triphenyl-tetrazolium chloride (TTC) added to soil extract agar.

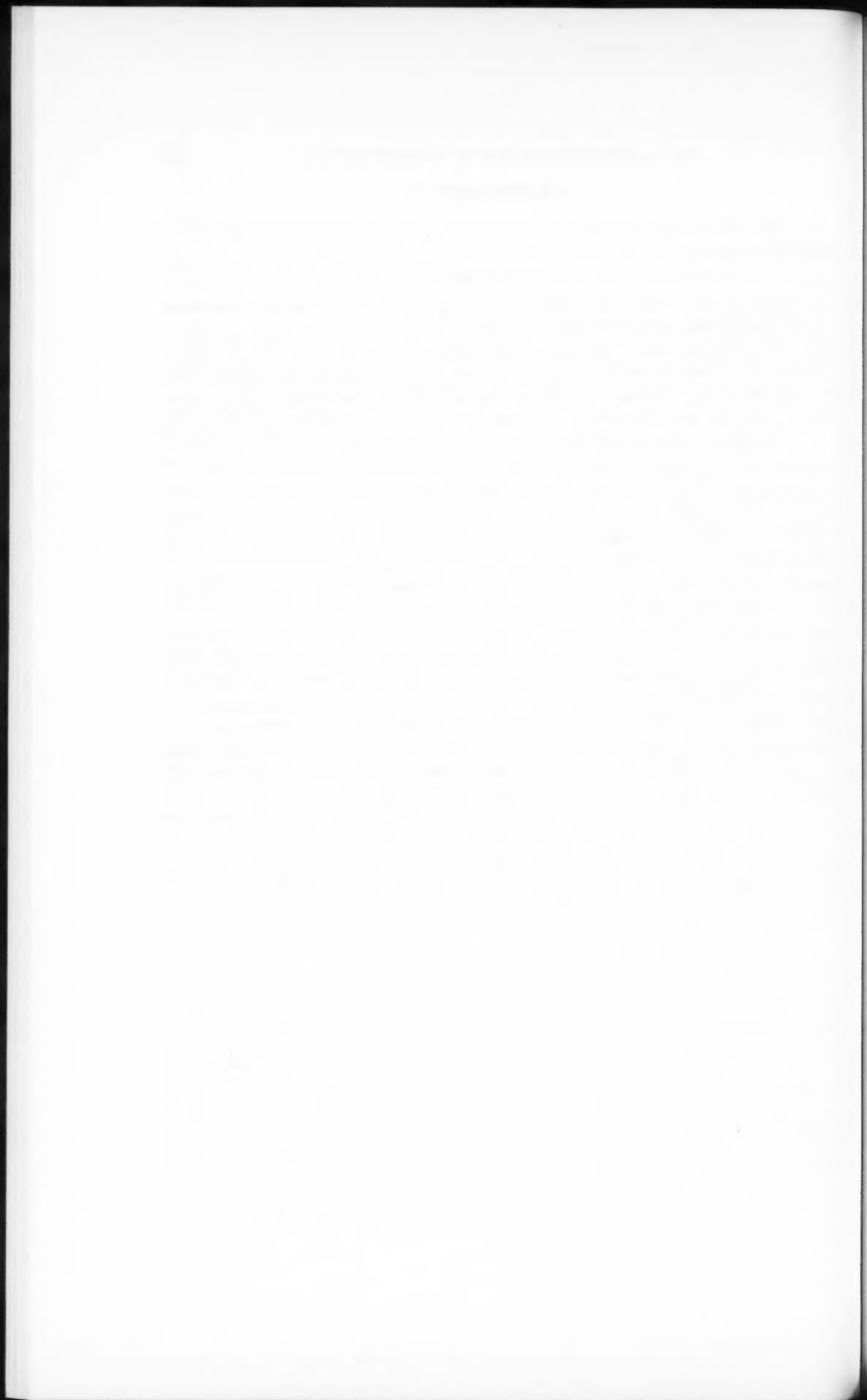


### Acknowledgment

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## THE COMPARATIVE GROWTH OF BACTERIAL ISOLATES FROM RHIZOSPHERE AND NON-RHIZOSPHERE SOILS<sup>1</sup>

J. W. ROUATT AND H. KATZNELSON

### Abstract

The growth response of bacterial isolates from the rhizosphere of greenhouse and field crops and control soils was studied in media of increasing complexity. In general, a greater proportion of rhizosphere isolates from the greenhouse crops grew more abundantly than did those from non-rhizosphere soils. This was not evident in all cases with the field crops. The addition of root extracts to an already complex medium did not result in growth stimulation. In fact the extract from oat roots was decidedly inhibitory to both rhizosphere and soil isolates.

### Introduction

Soil is the habitat of one of the most complex of natural biological communities in which all the phenomena associated with the struggle for survival occur. This competition is greatly intensified in the root zone of plants, the rhizosphere, owing to the greater density of its micropopulation. From an ecological point of view, it might be expected that in a region of such intense activity there would emerge groups or types of organisms which by their capacity to resist the antagonistic influences of associated organisms and by their inherent ability to reproduce more rapidly, would gain ascendancy and become the predominant forms. Conversely other types would be reduced to a minor status or even be eliminated. It therefore became of interest to determine if the bacterial population of the rhizosphere did indeed consist, in general, of types which could multiply more rapidly than those from the control soil. That there is a higher percentage incidence of physiologically active types of organisms in the root zone has been reviewed briefly in another paper (1) in this issue. Rovira (6) using organisms isolated at random found that the rhizosphere contains bacteria which are able to develop more rapidly than those from control soil.

### Experimental Methods

Both greenhouse and field samples were used in these experiments. To obtain more uniform samples in the greenhouse, crops were sown in large rectangular tanks containing uniform soil to a depth of 18 in. These were kept suitably moistened and were considered much more suitable than pots for growing plants. Samples for analysis were obtained as described in previous publications (2, 5). One control sample served two rhizosphere samples. In the field, the control samples were taken midway between adjacent rows of the crops. The samples were plated with soil extract agar, and following an incubation period of 14 days at 26° C. all bacterial colonies

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on a plate or representative portion thereof were picked and transferred to soil extract semisolid agar. After a further incubation period of 5 days at 26°C. the nutritional grouping of the isolates was carried out by the procedure previously described (3) involving growth response in the following media:

Medium B — basal medium,

Medium A — basal medium + amino acids,

Medium AG — basal medium + amino acids + growth factors,

Medium Y — basal medium + yeast extract,

Medium YS — basal medium + yeast extract + soil extract.

This procedure served a dual purpose. It made possible the classification of the isolates according to their nutritional needs and thereby a determination of the relative incidence of these various nutritional types in the soils and rhizospheres. By the spectrophotometric determination of turbidity it also made possible an analysis of the amount of growth produced by all the cultures in five different media under identical conditions.

Additional studies included the effect of root extracts on the soil and on the homologous rhizosphere isolates. Root extracts were prepared by washing roots thoroughly in water and allowing them to dry overnight at room temperature. The roots of six plants were then remoistened in 150 ml. of distilled water, which resulted in the liberation of large amounts of ninhydrin-positive and reducing substances. The solution thus obtained was added to YS medium (10% v/v) and inoculated along with the above media. After 5 days' incubation at 26° C. turbidity was determined in a Luxtrol photoelectric colorimeter, using the uninoculated medium for the zero setting (100% transmission).

## Results

### *Incidence of Nutritional Groups in Soils and Rhizospheres*

The data presented in Table I are representative and were included merely to demonstrate that the rhizosphere exerted its usual preferential effect on amino-acid-requiring bacteria as has been reported previously from this laboratory (4).

TABLE I

NUTRITIONAL CLASSIFICATION OF ISOLATES FROM RHIZOSPHERE AND CONTROL SOIL

Nutritional group	Greenhouse crops, %			Field crops, %		
	Control soil	Barley rhizosphere	Pea rhizosphere	Control soil	Wheat rhizosphere	Oats rhizosphere
B	3.3	1.8	1.5	1.1	3.0	5.6
A	26.2	53.1	63.9	16.6	60.7	29.6
AG	31.9	23.0	20.8	35.4	13.1	36.3
Y	17.1	10.6	9.2	21.9	8.1	13.7
YS	21.3	11.5	4.6	25.0	15.1	14.8
Plate counts*	93	1429	2610	64	2124	602

\*Millions per gram oven-dried soil.

*Growth of Soil and Rhizosphere Isolates*

In these experiments no attempt was made to use plants of comparable age. Of the greenhouse crops, barley and peas were the youngest and tomatoes and beans the oldest. Wheat and oats obtained from the field were in the seedling stage, whereas rye and barley were near maturity. The results obtained are summarized in Table II. In general it is evident that a greater

TABLE II  
GROWTH OF SOIL AND RHIZOSPHERE ISOLATES IN THREE MEDIA\*

Crops	No. isolates tested	Test media		
		B	A	Y
Greenhouse				
Control soil	94	5	24	45
Barley rhizosphere	113	14	58	81
Pea rhizosphere	130	19	69	89
Control soil	108	8	29	41
Red clover rhizosphere	110	13	26	84
Flax rhizosphere	101	26	49	85
Control soil	50	10	26	58
Tomato rhizosphere	74	23	58	90
Bean rhizosphere	101	56	71	98
Field				
Control soil	96	29	43	82
Wheat rhizosphere	99	73	83	97
Oat rhizosphere	88	31	45	84
Control soil	107	23	46	68
Rye rhizosphere	105	13	25	91
Barley rhizosphere	104	58	71	88

\*Figures represent percentage of cultures showing less than 90% transmission in Luxtrol photoelectric colorimeter.

proportion of rhizosphere isolates grew more abundantly than did those from non-rhizosphere soil. This is particularly true for the greenhouse crops in the three test media used, and for two of the field crops (wheat and barley). It is interesting that no difference in growth was noted between isolates from the oat rhizosphere and those of the control soil. Another noteworthy feature is the relative decrease in the percentage of isolates capable of producing moderate to good growth in media B and A in the rye rhizosphere. In the more complex medium, Y, an increase occurred however, suggesting that these isolates may require certain substances in yeast extract for maximum growth.

The effect of the addition of root extracts of greenhouse and field plants on the growth of homologous isolates is shown in Tables III and IV respectively. Considering the greenhouse crops it is evident that all the isolates grew more abundantly, as would be expected, in medium YS, than in media B, A, and Y (Table II). For this reason the increase in the number of rhizosphere isolates showing less than 90% transmission is not so striking.

TABLE III

COMPARISON OF THE GROWTH OF ISOLATES FROM THE RHIZOSPHERE OF GREENHOUSE PLANTS AND FROM CONTROL SOIL IN RELATION TO ROOT EXTRACTS\*

Medium	Control soil	Rhizosphere					
		Barley	Pea	Red clover	Flax	Tomato	Bean
YS	75	87	96				
YS + barley extract	84	97	—				
YS + pea extract	83	—	97				
YS	59			99	96		
YS + red clover extract	54			91			
YS + flax extract	62				92		
YS	82					93	99
YS + tomato extract	82					89	
YS + bean extract	80						96

\*Percentage of cultures showing less than 90% transmission in Luxtrol photoelectric colorimeter.

TABLE IV

COMPARISON OF THE GROWTH OF ISOLATES FROM THE RHIZOSPHERES OF FIELD CROPS AND FROM CONTROL SOIL IN RELATION TO ROOT EXTRACTS\*

Medium	Control soil	Rhizosphere	
		Wheat	Oats
YS	85	100	97
YS + wheat extract	83	100	
YS + oat extract	6		4

\*Percentage of cultures showing less than 90% transmission in Luxtrol photoelectric colorimeter.

However, the same trend as noted in Table II also occurs, that is, an increase in the number of rapidly growing forms, to a greater or lesser degree, in the rhizosphere of all the plants tested. The addition of root extracts did not appear to alter these results. This may be due again to the adequacy of medium YS in fulfilling all the nutritional demands of the isolates tested. A more striking effect of these extracts might have occurred if they were added to simpler media (B, A, or Y). The age of the plants from which these extracts are obtained may also be important in this connection. The results obtained with the field crops are limited but nonetheless interesting. Wheat isolates in media YS and YS plus wheat root extract showed the same trend as that obtained with the greenhouse crops, that is, a small relative increase of rapidly growing bacteria in the rhizosphere. On the other hand, the addition of oat root extract to medium YS resulted in a marked inhibition of the corresponding isolates.



### Discussion

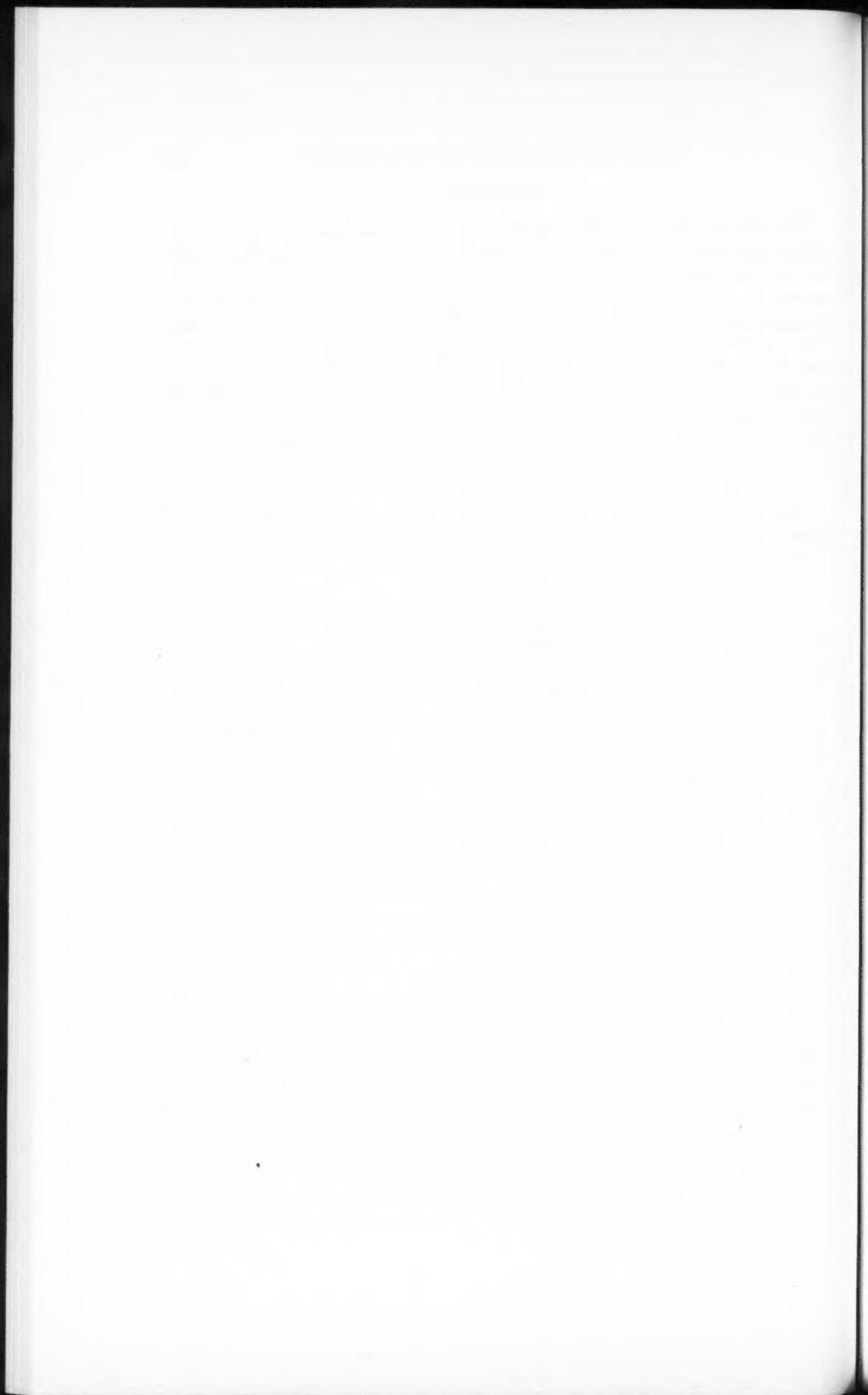
The results are of significance from an ecological point of view. They reflect the competitive forces occurring in the rhizosphere, resulting finally in the predominance of more actively growing bacteria. It has also been shown in the preceding paper (1) that the rhizosphere harbors bacteria that are more active physiologically than those from non-rhizosphere soil. This active bacterial mantle may play an important role in the nutrition and well-being of the plant. It may contribute to the resistance of the plant to soil-borne diseases or to its supply of nutrients. Conceivably it may be detrimental and render the root more susceptible to pathogenic forms. Studies dealing with these aspects of the rhizosphere problem are being planned.

### Acknowledgment

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## THE POLYSACCHARIDE PRODUCED BY AZOTOBACTER INDICUM<sup>1</sup>

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### Abstract

A procedure was developed for the isolation and purification of the polysaccharide produced by *Azotobacter indicum* which resulted in a product containing approximately 5% ash, 0.4–0.6% nitrogen, and 40% carbon. Qualitative tests showed the absence of galactose, pentoses, ketoses, and amino and methylated sugars, and the presence of glucose and a uronic acid. A technique for determining combined uronic acids resulted in a value of 30% uronic acid on the untreated (unhydrolyzed) polysaccharide, and the remaining components were determined by analysis of the material after acid hydrolysis. The relatively severe conditions required to hydrolyze the polysaccharide indicate that the components are present in the pyranose form. The results obtained when the hydrolyzates were analyzed by paper chromatography, the spectrophotometric reactions of Dische, and other specific quantitative assays showed that the polysaccharide is a polymer of glucose, glucuronic acid, and an aldoheptose in the ratio of 3 : 2 : 1. Infrared analysis confirmed the presence and relative proportion of the uronic acid residues and indicated that the sugar units of the polysaccharide are probably in the beta configuration.

### Introduction

Bacterial polysaccharides have received considerable attention because of their striking physical properties, their immunological reactions, and their diversity of composition and structure. In addition to the formation of relatively simple polymers containing a single type of sugar, bacteria are known to synthesize complex polysaccharides consisting of several types of sugars and frequently also amino sugars and uronic acids. The wide variety of these polysaccharides has been reviewed by Evans and Hibbert (11).

More recently, heptoses have been reported as constituents of certain polysaccharides. For example, Jesaitis and Goebel (14) found that the specific polysaccharide of *Shigella sonnei* contained 20% heptose. Aldoheptose sugars were reported to be present in smaller quantities in the polysaccharide material from *Shigella flexneri* (21) and *Shigella dysenteriae* (5), and as a major component in *Chromobacterium violaceum* (4). Davies (4) suggested that different groups of sugars may be characteristic of different taxonomic groups of bacteria.

The studies on azotobacter gums have dealt largely with that produced by *Azotobacter chroococcum*. Cooper, Daker, and Stacey (3) reported that the polysaccharide of *A. chroococcum* consisted of 87% glucose and 3% uronic acid. From the recognition of glucose and uronic acid as constituents, the authors placed the gum in the same class of compound as the immunologically specific polysaccharide of certain pneumococcal types. Both the polysaccharide of *A. chroococcum* and that of *Rhizobium radicicolum* (authors' nomenclature), which was similar, gave cross precipitin reactions with pneumococcal

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Contribution from the Department of Bacteriology, University of Wisconsin, Madison, Wisconsin.

antiserum. Later, using chromatographic methods of analysis, Lawson and Stacey (15) found that the polysaccharide contained not only glucose and glucuronic acid, but also galactose and a trace of a dideoxy sugar.

Martin (17) has reported that *A. indicum* produced a dextran when grown on glucose. In the present study, preliminary tests indicated that the polysaccharide isolated from the culture of *A. indicum* used was not a true dextran, *i.e.*, a gum containing only glucose units. Applying chromatography and other more recently developed techniques, the composition of this polysaccharide has been investigated.

### Methods

#### *Isolation and Purification of the Polysaccharide*

For production of the polysaccharide, cells were grown in twenty 500 ml. shake flasks, each containing 100 ml. medium, with glucose as the carbon source. This was changed to larger scale production in which 3-liter volumes were used when a New Brunswick stirred jar fermentor became available.

The culture was harvested after approximately 3 days (when there was no further increase in turbidity) and the cells were removed by centrifugation of the liquid three times in a refrigerated Sharples clarifier at 50,000 r.p.m. The supernate was adjusted to pH 7.5 and filtered through glass wool into cold 95% ethanol, which resulted in the precipitation of the polysaccharide as a crude gelatinous material. This product was purified by alternate solution in water and precipitation with cold ethanol several times. The material was difficultly soluble in water and could be redissolved only by being heated in water in a steamer or autoclave for several minutes. Following the treatment with 95% ethanol, the polysaccharide was precipitated from smaller volumes of water with acetone or absolute ethanol, washed with ether several times, and dried in a vacuum desiccator. As the resulting fibrous product was hygroscopic, it was stored in a desiccator over Drierite. A yield of approximately 1.5 g. of the purified polysaccharide was obtained from 1 liter of culture fluid.

#### *Hydrolysis Procedures*

For hydrolysis of the polysaccharide, solutions of sulphuric and oxalic acids were used with varying times and temperatures. The specific figures will be given where they apply in the text. The extent of hydrolysis was measured by determining the difference in reducing sugars present before and after hydrolysis, using the Nelson colorimetric adaptation of the Somogyi procedure (19). Following the hydrolytic treatment the solutions were neutralized with barium hydroxide and the insoluble barium salt of the acid removed by centrifugation. The supernate was treated with a cation exchange resin to remove excess barium and then concentrated to a small volume under reduced pressure. Solutions prepared in this way were used for chromatographic analyses.

### *Chromatographic Procedures*

For paper chromatography, a 460 × 150 mm. glass cylinder was used. The samples were applied to Whatman No. 1 filter paper with a small platinum loop and allowed to air dry. The cylinder with the paper suspended in it was sealed either with a plate glass cover and plasticine or with Saran Wrap (Dow Chemical Company). The latter was found to be a more rapid and efficient means of obtaining an airtight seal.

Acidic (butanol, acetic acid, and water in a ratio of 4 : 1 : 5; ethyl acetate, acetic acid, and water in a ratio of 9 : 2 : 2), basic (butanol, pyridine, and water in a ratio of 10 : 3 : 3), and neutral (butanol, ethanol, and water in a ratio of 4 : 1 : 5) solvents were used. For detection of compounds the aniline hydrogen phthalate spray of Partridge (20) was used. The movements of the unknown samples were compared with those of known compounds which were run simultaneously.

### *Identification of Components*

The methods for identification of the individual components of the polysaccharide will be described as they are discussed under Results.

## **Results**

### *Identification of Constituents in Gum*

Only a limited number of analytical determinations can be made on untreated (unhydrolyzed) polysaccharides, e.g., for ash, nitrogen, carbon, methoxyl, and uronic acid and a few qualitative tests for certain sugars. The polysaccharide prepared as above contained 5% ash, 0.4–0.6% nitrogen, and approximately 40% carbon. Qualitative analysis indicated the absence of galactose, pentoses, ketoses, amino sugars, and methylated sugars, and the presence of glucose and a uronic acid.

Estimation of the uronic acid was one of the few quantitative analyses which could be made on the polysaccharide without prior hydrolysis. This test is based on the fact that when a uronic acid is heated with hydrochloric acid, it is decomposed to furfural, carbon dioxide, and water, the yield of CO<sub>2</sub> being quantitative (6). The method used on the polysaccharide of *A. indicum* was an adaptation of the semimicro method of Maher (16). In place of the apparatus described by Maher, the wet carbon oxidation apparatus of Van Slyke was used. The sample plus 12.5% hydrochloric acid was placed in a 100 ml. round bottom flask connected to the apparatus by a ground glass joint and heated at 150° C. for 4 hours. The carbon dioxide evolved was trapped in barium hydroxide and measured by titration at the end of the heating period. Reagent blanks and standard solutions of glucuronic acid were treated in the same way. The resulting value of 30% uronic acid from duplicate analyses of the gum was in agreement with that obtained by the naphthoresorcinol colorimetric assay of Fishman and Green (12), which gave an average of 31% uronic acid when applied to three polysaccharide preparations isolated at different times.

For further analysis, it was necessary to hydrolyze the polysaccharide by the action of hot dilute acid solutions. The polysaccharide of *A. indicum* appeared to be partially resistant to hydrolysis in that the maximum reducing value obtained was 60% using 0.75 *N* sulphuric acid for 1 hour at 120° C. Hydrochloric and oxalic acids were not as effective as sulphuric. The difficulty in hydrolyzing the polysaccharide may reflect the presence of pyranose ring structures. When a furanose form of a sugar is contained in a glycoside, the linkage is quite easily split; for example, with a uronic acid-sugar glycoside, even the acidity of the free gum in a boiling water solution is enough to sever such a bond. However, a pyranoside survives this mild treatment, and its complete degradation may require 2 to 4% acid at 120° C. for several hours, during which time destruction of the constituents, especially uronic acids, may occur (1). Such destruction was observed by noting the absorption of the solutions at 280–290  $\mu$ , the wave length at which sugar breakdown products (furfurals) absorb (21); the amount of destruction varied with acid concentration, temperature, and time of hydrolysis. Attempts to hydrolyze the polysaccharide enzymatically, which would free the components without degradation, using amylase, emulsin, and glucuronidase, were unsuccessful.

The reducing powers of the acid hydrolyzates were calculated using glucose in the preparation of the standard curve. However, glucose assayed by the glucose oxidase procedure accounted for only 45% of the reducing power. Knowing that glucuronic acid was also a constituent, the relative reducing capacities of standard solutions of glucuronic acid were determined, and it was found that glucuronic acid displayed 80% of the reducing capacity of an equivalent amount of glucose in the range 100 to 350  $\mu$ g. Therefore, the reducing values for the hydrolyzates obtained by comparison with the glucose standard curves were low.

To determine if other products were present which might also affect the reducing value, paper chromatograms were made of the hydrolyzates. Three spots were observed; one which corresponded to glucose, one which corresponded to uronic acid, and a third, unidentified component. The aniline hydrogen phthalate spray produced a yellow color with the uronic acid, a brown color with glucose, and pink to purple with the third component. The movement of the latter compound did not correspond to that of any hexose or pentose tested.

The first information concerning the identity of this compound resulted from analysis by the cysteine color reactions of Dische (9). These spectrophotometric reactions for the microdetermination of polysaccharide constituents are based on the heating of the materials with strong acid to form furfural derivatives and subsequent condensation of the furfurals with aromatic phenols, amines, and certain sulphhydryl compounds. The spectra were determined using the Beckman DU spectrophotometer. For every unknown and standard sample to which the color developer, in this reaction cysteine, was added, a duplicate in which the cysteine was omitted was run. The



absorption of the samples lacking cysteine was subtracted from the corresponding samples with cysteine, thus eliminating any color not resulting from the reaction of the sample and developer, i.e., developed with the acid alone.

The spectrum obtained with a gum hydrolyzate in the general cysteine reaction had an absorption maximum at  $510\text{ m}\mu$  which was not observed with glucose or glucuronic acid. Non-hydrolyzed gum also exhibited this  $510\text{ m}\mu$  peak. According to Dische (8), such an absorption pattern is typical of heptose sugars, which also display a characteristic absorption maximum at  $400\text{--}405\text{ m}\mu$  from the action of sulphuric acid alone. With sulphuric acid, hexoses show absorption maxima between  $305\text{--}330\text{ m}\mu$  and their absorption at  $400\text{ m}\mu$ , where the breakdown product of heptoses has its maximum, is negligible. After the addition of cysteine to the reaction mixture, new compounds are formed which also have characteristic absorption patterns. The reaction product of hexoses with a maximum at  $412\text{--}414\text{ m}\mu$  is formed rapidly and then slowly transformed to a substance with an absorption maximum at  $600\text{ m}\mu$ . The reaction product of heptoses, produced immediately after addition of cysteine, has an absorption maximum around  $460\text{ m}\mu$  which slowly shifts to a maximum at  $510\text{ m}\mu$ .

To test the validity of the results obtained with cysteine, two other specific colorimetric reactions of heptoses were applied: the orcinol reaction and the diphenylamine reaction (8). The reaction with diphenylamine and hydrochloric acid results in the formation of colored compounds with glucose and glucuronic acid as well as the heptose, but the wave lengths at which they absorb differ. The hexose solution shows absorption maxima at  $630\text{ m}\mu$  and  $520\text{ m}\mu$  with a minimum at  $560\text{ m}\mu$ , where the heptose solution has its maximum.

The second reaction is based on the fact that heptoses produce with orcinol in dilute hydrochloric acid two characteristic colored compounds with different absorption spectra. The first compound with an absorption maximum at  $590\text{ m}\mu$  is formed from the heptose solutions after 3 minutes' heating with orcinol in hydrochloric acid. When the mixture is diluted with double its volume of glacial acetic acid, the absorption maximum shifts to  $610\text{--}615\text{ m}\mu$ . No absorption is observed with glucose or glucuronic acid.

These tests were repeated several times on different gum preparations and different hydrolyzates to eliminate the possibility of anomalous absorption peaks resulting from techniques used in the preparation and purification of the gum or from varying hydrolytic procedures. The possibility still existed, however, that some compound might be formed from interaction of the materials during hydrolysis which could give rise to the colored products obtained with the various organic developers. To test this, solutions of (1) glucose and glucuronic acid, (2) glucose, glucuronic acid, and heptose, and (3) the polysaccharide were heated at  $120^\circ\text{C}$ . in  $1\text{ N}$  sulphuric acid for approximately 60 minutes. Each solution was neutralized with sodium hydroxide and analyzed by the procedures described above. Figs. 1, 2, and 3 show the results of these analyses made on the three similarly treated solutions.

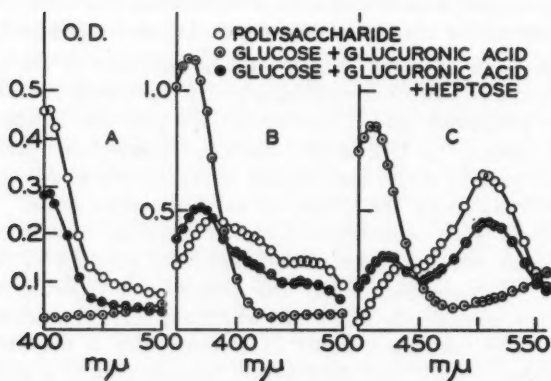


FIG. 1. The absorption spectra of solutions of (1) glucose and glucuronic acid; (2) glucose, glucuronic acid, and heptose; and (3) polysaccharide in the general cysteine reaction of Dische. The three solutions were similarly treated with an acid hydrolysis procedure prior to the test: A, before cysteine; B, 30 minutes after cysteine; C, 20 hours after cysteine.

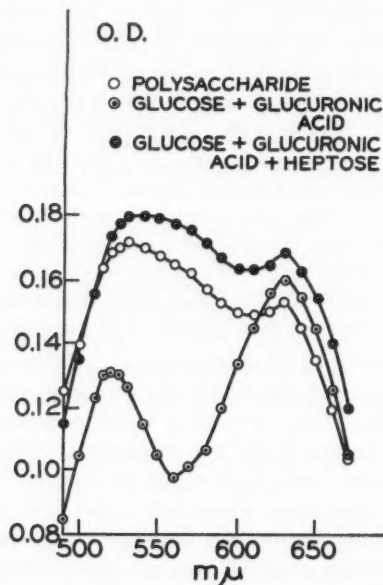


FIG. 2. The absorption spectra of solutions of (1) glucose and glucuronic acid; (2) glucose, glucuronic acid, and heptose; and (3) polysaccharide in the diphenylamine reaction. The three solutions were similarly treated with an acid hydrolysis procedure prior to the test.

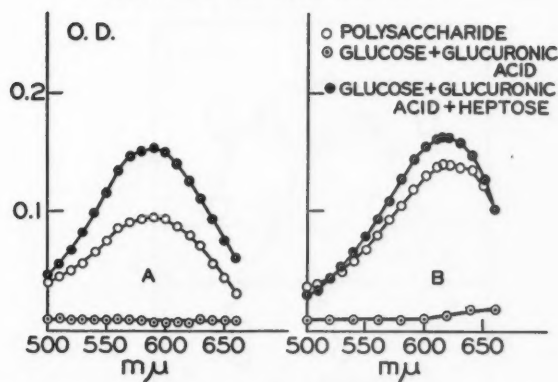


FIG. 3. The absorption spectra of solutions of (1) glucose and glucuronic acid; (2) glucose, glucuronic acid, and heptose; and (3) polysaccharide in the orcinol reaction. The three solutions were similarly treated with an acid hydrolysis procedure prior to the test: A, before the addition of acetic acid; B, after the addition of acetic acid.

The forms of the polysaccharide hydrolyzate absorption curves in the three procedures are nearly identical with those observed for the solution containing glucose, glucuronic acid, and glucoheptose.

When paper chromatograms were again run with the foregoing results in mind, it was found that the previously unidentified spot had the same movement as a standard glucoheptose run simultaneously. The known heptose also exhibited a pink color with the aniline hydrogen phthalate spray. When the papers were treated with a specific ketoheptose spray (2), no reaction was observed. Also, as mentioned earlier, the Seliwanoff test for ketoses was negative when applied to the polysaccharide. Thus, the heptose component of the gum is not a ketose but an aldose sugar.

Fig. 4 shows the movements of these materials in basic and neutral solvent systems. By allowing the neutral solvent to traverse the paper once and, after air-drying, reirrigating the paper in the same solvent system, the separation of the components was improved. This is shown in Fig. 4B.

With an acidic solvent, the movement of the uronic acid was increased, and the three components were poorly resolved. However, it was useful in that it caused one uronic acid, glucuronic, to lactonize, and the rapidly moving lactone (anhydro) compound could be readily distinguished from the other components. The glucuronic acid lactone spot was observed with standard glucuronic acid solutions and with the hydrolyzate sample on papers treated with the acidic solvent. This lactonization has been reported to occur only with glucuronic acid and not with galacturonic or mannuronic acids (13).

To confirm this identification, a colorimetric procedure for differentiating between the three hexuronic acids was used, dependent upon their reaction with thioglycolic acid in the presence of mannose (7). The results of this reaction are shown in Fig. 5. The barium uronate fraction of the polysaccharide was prepared from a sulphuric acid hydrolyzate, which had been

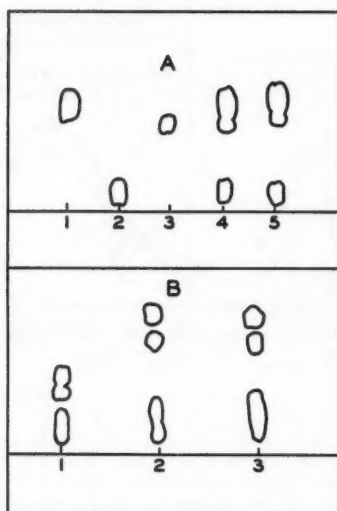


FIG. 4. Diagrammatic representation of paper chromatograms.

A. Butanol, pyridine, water solvent in the ratio 10 : 3 : 3 at room temperature: 1, glucose; 2, glucuronic acid; 3, glucoheptose; 4, mixture of 1, 2, and 3; 5, polysaccharide hydrolyzate.

B. Butanol, ethanol, water solvent in the ratio 4 : 1 : 5 at room temperature: 1, glucose, glucuronic acid, and glucoheptose after one development in the solvent; 2, the same components after two developments in the solvent; 3, polysaccharide hydrolyzate after two developments in the solvent.

neutralized with barium hydroxide. After the barium sulphate was removed, the barium salt of the uronic acid was precipitated with 10 volumes of methanol. The results of this assay and the paper chromatograms indicate that the uronic acid component of the gum is glucuronic acid, which is the most commonly occurring uronic acid in bacterial polysaccharides.

The sugar constituents of some bacterial polysaccharides have been reported to be present as phosphate esters. For example, Slein and Schnell (22) isolated an aldoheptose phosphate from the polysaccharide of *Shigella flexneri*. When their procedure for the isolation of the phosphate ester fraction was followed with the polysaccharide of *A. indicum* and the fraction assayed by paper chromatography and the colorimetric reactions of Dische, the results indicated that none of the constituents of the polysaccharide were present as phosphate esters.

#### Quantitative Analysis

The orcinol and diphenylamine reactions can be used for the quantitative determination of heptose in the presence of other sugars. In the diphenylamine reaction (see Fig. 2) calculations from the optical density at 560  $m\mu$  result in a value of 16% heptose in the gum solution. This reaction is less sensitive than the orcinol reaction (see Fig. 3), where the difference in the optical densities at 610 and 530  $m\mu$  is used to measure the concentration of

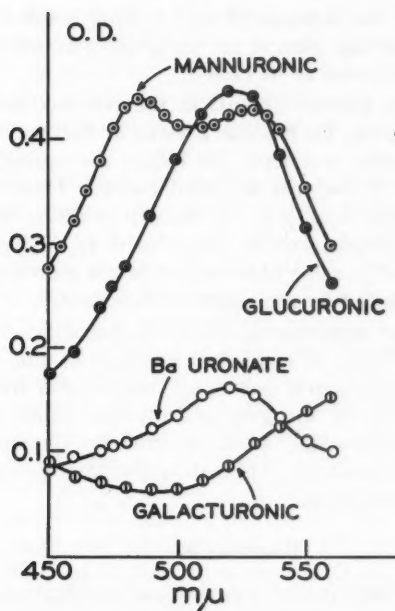


FIG. 5. The absorption spectra of solutions of glucuronic, galacturonic, and mannuronic acids and the methanol insoluble barium uronate fraction from an acid hydrolyzate of the gum in the thioglycolic acid test of Dische.

heptose, thus eliminating interference from other sugars. Comparison of the density increments of the gum solution and the standard heptose solution result in a value of 15% heptose in the polysaccharide.

To determine the amount of glucose present, the anthrone procedure described by Mokrasch (18) was used. Mokrasch stated that neither uronic acid nor glucoheptonate produced more than 4% of the color of equivalent amounts of glucose in the reaction. When this method was used in the present study, no color was observed with glucuronic acid in concentrations up to 150  $\mu\text{g.}$ ; however, glucoheptose gave slightly over 50% of the color of an equivalent amount of glucose. The three solutions of glucose - glucuronic acid, glucose - glucuronic acid - heptose, and polysaccharide which had been similarly treated in the hydrolytic procedure were analyzed by this anthrone adaptation.

For the glucose (38  $\mu\text{g.}$ ) plus glucuronic acid (16  $\mu\text{g.}$ ) sample, an average optical density of 0.310 was observed, which corresponds to a value of 38  $\mu\text{g.}$  on the glucose standard curve.

For the glucose (62.5  $\mu\text{g.}$ ), glucuronic acid (37.5  $\mu\text{g.}$ ), and glucoheptose (25  $\mu\text{g.}$ ) sample calculation, the optical density corresponding to 25  $\mu\text{g.}$  of heptose was read from the heptose standard curve (0.12) and subtracted from

the observed density of the solution (0.62), leaving a value of 0.50. This value is equivalent to 63  $\mu$ g. glucose on the glucose standard curve, which agrees with the amount known to be present.

In the same way, the glucose present in the polysaccharide sample was calculated. From the value for heptose present in the gum obtained in the orcinol and diphenylamine reactions (15–16%), the corresponding optical density was read from the heptose standard curve. This value subtracted from the observed optical density is the density resulting from the glucose in the sample. The calculations result in a value of 45–46% glucose. This is in agreement with the 45% value obtained when the glucose present in gum hydrolyzates was determined by the glucose oxidase assay.

In the procedure for determining reducing capacity, the aldoheptose showed only 75–80% of that of equivalent amounts of glucose in the range of 75 to 250  $\mu$ g. As mentioned previously, this is also true of glucuronic acid. The reducing value of the gum hydrolyzate, which was 68% when calculated as glucose, became 90% when adjusted for the three constituents present in their correct amounts: approximately 45% glucose, 30% glucuronic acid, and 15% aldoheptose.

### Discussion

From the procedures used in the isolation and purification of the polysaccharide of *Azotobacter indicum*, a product resulted containing approximately 5% ash and from 0.4 to 0.6% nitrogen, which was not present as an amino sugar constituent. A number of bacterial polysaccharides have been reported to contain up to 1% non-amino sugar nitrogen, the removal of which is very difficult and usually involves degradation of the polysaccharide. Stacey (23) considers this nitrogenous constituent as a "vestigial remain" originating from the nucleoprotein of the enzymes responsible for synthesis of the polysaccharide.

Examination of this purified product showed that it contained a uronic acid which was quantitatively determined on non-hydrolyzed material and found to account for 30% of the polysaccharide. This is considerably higher than the uronic acid content (3%) of the polysaccharide of *A. chroococcum* and slightly higher than the value (23%) reported for *Rhizobium radicicolum* (3).

In addition to the quantitative assays for uronic acid reported in the results, the infrared spectrum of the *A. indicum* polysaccharide was investigated and the presence of a strong absorption peak at 1600  $\text{cm}^{-1}$  confirmed the presence and the magnitude of the carboxylate ion arising from the uronic acid residues in the polysaccharide. Lawson and Stacey (15) reported that the *A. chroococcum* polysaccharide displayed only weak absorption in this region. The infrared spectrum of the *A. indicum* polysaccharide also showed the absence of absorption at 840  $\text{cm}^{-1}$  and the presence of peaks of moderate intensity at 890–900  $\text{cm}^{-1}$ , indicating that the sugar units are probably in the beta configuration (15).



Although Martin (17) reported that *A. indicum* produced a dextran when grown on glucose, the present studies have revealed the presence of glucuronic acid and an aldohexose in addition to the glucose component. Similarly, Lawson and Stacey (15) found galactose and a dideoxy sugar in the polysaccharide of *A. chroococcum* which had been previously identified as a glucose-glucuronic acid polymer (3).

The polysaccharide isolated from *A. indicum* consisting of glucose, glucuronic acid, and heptose does not appear to be utilized by the organism in the purified form. This may be the result of alteration of the material during the isolation process from its composition in the "native" state. However, in many instances such alteration appears to be minor, if it does occur; for example, the purified polysaccharide of the pneumococci will react with antibodies produced by whole cells. Conversely, the reason for the lack of utilization may be that the polysaccharide does not function as a storage product, but rather as a protective covering or as an accumulation of waste products. Dubos (10) suggests that all capsular polysaccharides are waste products and sustains his view by pointing out the results of the studies concerning the metabolic origin of dextrans and levans of certain bacteria in which high molecular weight polysaccharides are formed from the residue of the carbohydrate substrate which the organism does not utilize.

Whether waste material, protective covering, or storage material, the polysaccharide of *A. indicum* constitutes a major metabolic product and, as such, is of interest in any study of the physiology of the organism.

### Acknowledgments

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## MICROORGANISMS IN THE RUMEN OF CATTLE FED A CONSTANT RATION<sup>1</sup>

R. E. HUNGATE

### Abstract

Direct microscopic and culture counts were made on samples of rumen contents obtained by stomach tube from each of 25 cows at monthly intervals during 3 months. The ration was timothy hay plus various amounts of cottonseed meal, rolled barley, and salt. No correlation between different rations and differences in the microbiota could be detected. *Streptococcus bovis* was identified by its rapid growth in the feed medium and showed counts ranging between  $2 \times 10^8$  and  $1.4 \times 10^8$ . Most animals showed a *S. bovis* count between 1 and 20 million with an average of about 10 million per milliliter. The characteristics of some rumen strains are described. The cellulose-digesting bacteria were referable to four chief groups and the numbers of each were recorded. The cellulolytic cocci varied in almost every character studied. Two types were distinguished as representing the greatest divergence. *Ruminococcus albus* n. sp. differs from *R. flavefaciens* in being Gram-negative, forming little or no yellow pigment, and producing no succinic acid. A cellulose-digesting sporeformer, *Clostridium lochheadii* n. sp., was found in many of the samples. It was extremely active in digesting cellulose, exceeding in this respect the species of anaerobic cellulose digesters previously isolated. Spores were formed in abundance but rapidly disintegrated and many strains were lost before subculture. *Clostridium longisporum* n. sp. was encountered during the investigation and is described.

"In den günstigsten Fällen wurde schon innerhalb eines Tages (bei 38°C.) kräftige Zersetzung (of cellulose) erreicht. Die Resultate stimmen also hier mit jenem überein, wie sie speziell im Verdauungskanal der Wiederkäuer beobachtet wurden, wie denn auch das Anhäufungsverfahren selbst sich den dort gegebenen Verhältnissen möglichst eng anschlieszt." (Löhnis and Lochhead 1913 (12)).

Dr. Grant Lochhead was not only early concerned with methods for demonstrating cellulolytic bacteria, he also appreciated the uniqueness of the rumen habitat and its pre-eminent fitness for rapid digestion of cellulose. For this reason and in recognition of Dr. Lochhead's lifelong interest in microorganisms as they exist in nature, the author takes great pleasure in contributing in his honor an account of some of the characteristics of certain microorganisms from the rumen.

During the summer of 1951, the Department of Animal Husbandry at the State College of Washington tested the effects of sodium chloride used to limit the feed consumed (4). During 4 months 25 pregnant range cows were fed eight different combinations of chopped timothy hay, cottonseed meal, rolled barley, and salt. They were available for a study of the rumen microorganisms.

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### Methods

The rations fed are shown in Table I.

Rumen contents were sampled at four different periods, June 15, July 5 and 6, August 3 and 4, and September 7 and 8. Each sample was removed through a stomach tube and, after it was mixed, 10 ml. was added to 90 ml. of salt solution in equilibrium with 5% carbon dioxide in a screw-capped bottle. The dilution fluid had the following composition: NaCl 0.6%,  $\text{KH}_2\text{PO}_4$  0.1%,  $\text{MgSO}_4$  0.01%,  $\text{CaCl}_2$  0.01%. After sterilization, 0.06%  $\text{Na}_2\text{CO}_3$  was added and the solution equilibrated with 95% nitrogen - 5% carbon dioxide. As soon as the samples were returned to the laboratory, air was again displaced with the  $\text{N}_2\text{-CO}_2$  mixture and the diluted materials were left undisturbed until just prior to inoculation when each sample was shaken vigorously for 30 seconds on a shaker having 600 oscillations per minute with an excursion from center of 8 mm. Further dilutions were made in the same diluent and inoculated into rumen fluid cellulose agar and feed extract agar series. The inoculated tubes were rolled as they solidified and were incubated at 39° C.

The rumen fluid cellulose agar medium contained 1% finely divided filter paper cellulose, wet ground in a pebble mill, and 30% rumen liquid obtained by squeezing rumen contents through cotton backed by cheesecloth. Ingredients of the medium in percentages were:  $\text{KH}_2\text{PO}_4$  0.02,  $\text{K}_2\text{HPO}_4$  0.03,  $\text{MgSO}_4$  0.01,  $\text{CaCl}_2$  0.01, NaCl 0.1,  $(\text{NH}_4)_2\text{SO}_4$  0.1, and resazurin 0.0001. Cysteine (0.02%) and sodium bicarbonate (0.5%) were added after sterilization and the mixture equilibrated with an atmosphere of carbon dioxide.

The feed extract medium was composed of 50 ml. of hay extract (4 g. timothy hay boiled 5 minutes in 100 ml. tap water and filtered), 25 ml. cottonseed meal extract (2 g. boiled 5 minutes in 100 ml. water), and 25 ml. barley extract (2 g. boiled 5 minutes in 100 ml. water). Other constituents were the same as for the rumen fluid cellulose agar except that the  $(\text{NH}_4)_2\text{SO}_4$ , NaCl, cellulose, and rumen fluid were omitted.

TABLE I  
EXPERIMENTAL RATIONS

Ration No.	Amount of timothy hay fed (as fraction of total digestible nutrients)	Kind of concentrate and amount (as fraction of total digestible nutrients)	Amount of NaCl (lb. per day)
1	2/3	Cottonseed meal 1/3	0.3
2	2/3	Cottonseed meal 1/3	Ad libitum
3	2/3	Rolled barley 1/4	0.3
4	2/3	Cottonseed meal 1/12 Rolled barley 1/4	Ad libitum
5	3/4	Cottonseed meal 1/12	
6	3/4	Cottonseed meal 1/4	0.2
7	8/9	Cottonseed meal 1/4	Ad libitum
8	8/9	Cottonseed meal 1/9	0.1
		Cottonseed meal 1/9	Ad libitum

On June 15 all animals were sampled and cultured on the same day. Sampling required about 2 hours and inoculations were not completed until 10 hours after the last sampling. During this time the anaerobic conditions kept the bacteria viable and the lower temperature and lack of added substrate reduced changes in numbers and kinds of organisms due to growth and death. In the June 15 series the order of inoculation and the culture counts were compared to detect any correlation, but none was found. For the remaining samplings, half of the animals were examined on one day and the other half on the next. This reduced the time between sampling and completion of culturing to 4 to 6 hours.

After 24 hours' incubation the feed agar tubes were examined for colonies of *Streptococcus bovis*, identified by their large size and the microscopic appearance of the cells. The tubes were further incubated and a total colony count made after 10 days. The cellulolytic colonies in the rumen cellulose agar were examined and counted after 20 days of incubation.

For direct counts of bacteria, 1/10 ml. of the 10X dilution was mixed with 9.9 ml. distilled water, and 0.01 ml. of this 1000X dilution spread evenly over 2 sq. cm. of a clean slide which had just previously been covered with a loopful of a 0.1% solution of sodium lauryl sulphate to facilitate even spreading. The smears were dried, fixed, and Gram stained. The bacteria were counted in a total of 15-20 microscope fields, evenly spaced across the smear on the vertical or horizontal mid-line. A greater concentration of organisms was always noted at the edge of the smear so four of the fields chosen were at the ends of the horizontal and vertical bisects. For the June 5th samplings no direct counts of bacteria were obtained.

For direct counts of protozoa, 1 ml. of the 10X dilution was added to 1 ml. of 40% ethanol, 8% acetic acid, 0.05% methyl green. The samples were left in this preservative until all had been collected. Counts were made using a mold counting chamber.

### Results of the Various Counts

The bacterial counts are shown in Table II. The direct microscopic counts ranged from a low of 8 billion per milliliter to a high of 47 billion. The total average count was 20 billion and the average for each of the three samplings did not differ significantly from this at the 5% level.

The total culture counts with the feed extract agar medium showed great variation. The averages for the different samplings differ significantly at the 1% level when tested by analysis of variance (16). The counts on June 15 for animals 44, 52ELO, and 59 were as high as many of those reported by other investigators for alfalfa hay rations. But the cultures from the other animals showed much smaller numbers. This variation may have been due to the shorter period (2 weeks) on the ration prior to sampling.

The feed extract medium apparently satisfied the culture requirements of only a small fraction of the bacteria in the rumen. The average total counts were only 0.1 to 1% of the direct count. Higher counts could undoubtedly

TABLE II  
NUMBERS OF BACTERIA AS ESTIMATED BY SEVERAL METHODS  
Counts in millions except direct count in billions

Cow No.	Ration No.	June 15 samples				July 5, 6 samples				August 3, 4 samples				September 7, 8 samples			
		Total culture	S. bowii	Cellulo-lytic bacteria		Total culture	S. bowii	Cellulo-lytic bacteria		Total culture	S. bowii	Cellulo-lytic bacteria		Total culture	S. bowii	Cellulo-lytic bacteria	
24	1	2.2	2.2	2		20	2	4		19	14	0.2		23	8	0.006	
35	1	120	4	0.24		44	6	3.2		19	8	10		20	2	0.6	
51	1	200	1.6	0.02		100	80	4.2		19	100	10		22	2.6	0.002	
45	2	200	4	48		36	12	4		28	240	10		31	10	0.8	
52WW	2	200	2	0.2		17	20	0.2		25	40	2		25	20	0.4	
42	3	4	3.6	4		25	18	4		28	30	2		13	30	0.16	
52ELO	3	1600	2	0.002		20	8	6		28	520	140		11	3	0.2	
61	3	2	1	0.2		16	3.8	0.06		47	60	22		8	2.4	0.48	
18	4	2	0.4	2		50	2.2	2.4		10	60	4		24	9.8	0.2	
42OK	4	1.7	0.6	0.14		22	4	0.2		14	160	6		22	2.4	0.04	
69	4	200	20	0.6		20	3.6	0.14		11	8	1.4		10	3.2	0.22	
57	5	300	2	2		26	4	4.1		35	120	16		11	6.6	0.22	
58	5	300	2	0.5		15	0.6	0.5		23	40	2		20	4	0.2	
66	5	380	0.8	0.04		3	0.6	0.5		21	18	8		13	12	0.1	
26	6	600	6	0.002		20	20	4.2		11	200	8		12	12	0.1	
46	6	200	0.2	0.002		45	4	2		13	140	14		32	22	0.08	
59	6	2200	20	0.002		18	24	2		11	470	10		9	5.2	0.26	
3	7	20	0.2	0.002		36	8	0.16		7	42	4		29	21	2.2	
43	7	400	8	0.002		22	40	6.4		14	100	10		19	3.2	0.06	
58	8	60	1.5	0.12		19	60	2		24	18	8		12	4	0.004	
44	8	3200	6	0.4		16	0.2	0.6		17	160	14		13	4	0.4	
60	8	800	2	0.04		34	3	0.44		12	180	14		9	10	0.006	
67	8	200	16	0.2		18	24	2		13	16	4		18.5	11	7.4	
Average		450	4.8	3.0		23.2	9.3	2.5		18.5	190	17.1		18.5	11	7.4	



have been obtained by using the improved techniques of Bryant and Burkey (1) and Maki (14). These include addition of rumen fluid to the medium, mixing the sample in a Waring blender, and adding cysteine to the dilution water. Even with improvements in culture techniques it is questionable whether the culture count would have constituted more than a few per cent of the direct count. According to Maki (14) the culture count for animals on a high fiber ration (alfalfa hay) is a much smaller fraction of the direct count than for animals on a low fiber ration. Timothy hay would be expected to contain a higher fiber content than alfalfa and might show an even greater discrepancy.

The numbers of *S. bovis* detected with the feed extract medium fell below 200,000 per milliliter in only 2 out of 93 samples. The largest count was 140 million per milliliter. The averages for each sampling period do not differ significantly at the 20% level when tested by analysis of variance. The culture counts for *S. bovis* are more consistent than either the total or the cellulolytic colony counts. They are about the same as the counts previously obtained for normal sheep (10) but are somewhat larger than the counts of Wilson (18).

The counts of cellulolytic bacteria were slightly lower than the counts for *S. bovis*. This probably does not indicate fewer numbers, since the cells of *S. bovis* are single or in twos whereas the cellulolytic bacteria accumulate on particles of plant material. Particularly since they were not dispersed with a Waring blender, the cellulolytic cell clumps would be relatively larger and less numerous. With the exception of the September samples the average values agree fairly well.

The September samples were immediately diluted to 1000 $\times$  (rather than 10 $\times$ ) and kept in this dilution until just before inoculation. In the 10 $\times$  dilution the organisms were sufficiently concentrated that traces of oxygen or other toxic factors were absorbed without damage during the storage period. In the 1000 $\times$  dilution this mutually protective effect was much less. The cells of *S. bovis* and others included in the total culture count are more resistant than the cellulose digesters and were less injured by this change in procedure. In many of the September samples the total culture count and the *S. bovis* count were the same.

In counting the protozoa, *Diplodinium* was identified and counted accurately. The average number was 6000 per milliliter of rumen contents, with a maximum of 27,000. The numbers did not vary with the ration. The factor for the direct count was 800. In many cases the number of *Isotricha* and *Dasytricha* per milliliter was below this value, so accurate average numbers were not obtained. The maximum count for *Isotricha* was 10,400 per milliliter and for *Dasytricha* 8800. If those animals showing fewer than 800 per milliliter were arbitrarily assumed to contain at least 400 per milliliter, the average counts for *Isotricha* and *Dasytricha* were 1200 per milliliter each. The *Entodinium* were considerably more numerous than any of the other protozoa but were not accurately counted.

### Characteristics of *S. bovis*

The colonies of *S. bovis* were white, light orange, or orange in color. Each of 48 colonies was suspended in a small quantity of sterile tap water and from this a loopful was inoculated into aerobic tubes of agar medium containing an inorganic solution plus rumen fluid and 0.5% starch. No reducing materials were added and cotton-plugged tubes were used. Incubation was at 45° C. No growth occurred in any of the tubes. It seemed possible that conditions in these cultures were too aerobic, so 18 of the same tubes were autoclaved, reinoculated, the air in the tubes replaced with nitrogen, and the tubes rubber stoppered. These tubes showed excellent growth the next morning. The results indicate that on initial isolation the strains were inhibited by oxygen.

The original colors were not maintained during subsequent transfers. Some of the white strains showed orange colonies and vice versa. The same medium was used in all tubes. Since in the original tubes both white and orange colonies had been found in the same regions, the color variations could not be accounted for by differences in the medium.

Eighteen strains of *S. bovis* were isolated from the cattle at Pullman, three from cattle at Ithaca, and six from sheep at Ithaca. Their characteristics are shown in Table III. Many of the sugars listed in Bergey's Manual as always fermented were not tested.

All strains were Gram-positive cocci. In fresh mounts they were often in pairs and then sometimes slightly lanceolate. Action on litmus milk was variable: 16 of the Pullman and two of the Ithaca strains from cattle formed acid with no reduction or coagulation, and two Pullman strains formed acid with slight coagulation but no reduction. The six sheep and one cattle strain from Ithaca showed acid, coagulation, and reduction. Most isolates caused no visible change in blood agar, but a few gave a very slight green color.

All strains grew readily in feed extract medium. It seemed probable that the starch in the barley was the fermentable substrate, yet the culture counts did not correlate with the amount of barley in the ration (Table IV). Rations 3 and 4 with the barley did not show significantly higher counts. The possibility that *S. bovis* might utilize other constituents was tested by incorporating them separately into anaerobic agar slants and inoculating with 11 of the Pullman strains. All showed growth and acid production within 24 hours on the hay extract and cottonseed meal extract, respectively, and 10 showed growth and acid production on the barley extract. Growth was not as profuse on the latter medium and in none of the tubes was growth as abundant as on the combined extracts. Before inoculation, only the barley extract medium gave a positive starch test. The results indicate that constituents of the ration other than starch can support growth.

Very high counts of *S. bovis* were observed in previous experiments (10) in which grain was suddenly administered to hay-fed sheep. In order to test whether continuous consumption of a high grain ration would cause high counts of *S. bovis*, rumen contents of a young bull on a fattening ration of

TABLE III  
CHARACTERISTICS OF STRAINS OF *S. bovis* ISOLATED  
FROM THE RUMEN OF CATTLE AND SHEEP

Fermentation characteristics	Strains from cattle				Strains from sheep			
	Pullman, 18 strains			Ithaca, 3 strains		Ithaca, 6 strains		
	+	-	±	+	-	+	-	±
Glucose	18			3		6		
Salicin	18			3		6		
Raffinose	18			3		6		
Lactose				3		6		
Cellobiose				2		3		
Starch	18			3		6		
Inulin	13	5		3		6		
Esculin	18			3		5	1	
Trehalose	8	10		1	2	6		
L-Arabinose	10	3	5		2	1	2	3
Mannitol	18				1	1	4	
D-Arabinose		18						
Sorbitol		18			3		6	
Glycerol		18			3		6	
D-Xylose		18			3		6	
Other characteristics								
Hydrolysis of Na hippurate		10			3		6	
Growth at 45° C.	16	1				5		
Survive 60° C. for 30 minutes	14	4						
Growth in 2% NaCl	18			3		6		
Growth in 6.5% NaCl		4			3		6	
Gelatin liquefaction		2						
Catalase		18			3		6	
Indol		10						
Capsule formation	18			2	1	2	1	
Urease		5						

TABLE IV

AVERAGE CULTURE COUNTS OF *S. bovis* ON DIFFERENT RATIONS, MILLIONS/ML.

Sampling	Rations (Table I)							
	1	2	3	4	5	6	7	8
June	2.6	3.2	2.3	0.7	8.3	8.7	4.3	8.4
July	29	8	4.7	3.9	4.2	9.3	6.1	9.1
August	10.7	12	55	7.8	19	12	7.3	10.7
September	4.2	16.9	4.8	3.9	8	22	3.3	5.7
Average	11.6	10.0	16.7	4.1	7.7	13	5.3	11.3

$\frac{2}{3}$  grain -  $\frac{1}{3}$  hay were inoculated into dilution series of wheat extract agar (filtrate from 1 g. ground wheat boiled in water and filtered through cotton). A *S. bovis* colony count of 5 million per gram rumen contents was obtained. Starch-digesting rods of various sorts were much more numerous. Repetition of the test gave a count of 7 million *S. bovis*. A continued high grain ration does not necessarily support unusually high numbers of *S. bovis*; other amylolytic organisms may outnumber it. A partial explanation for this

may be inability of *S. bovis* to attach to starch grains. Cells inoculated into liquid media containing raw starch showed no tendency to aggregate on the grains, in contrast to *Bacteroides amylophilus* (6) and the coccus of van der Wath (17).

The growth of *S. bovis* on hay suggested its presence in the hay before the latter was ingested. A sample of good quality grass hay and one of alfalfa hay were inoculated into a dilution series of rumen liquid glucose agar. No colonies of *S. bovis* developed, showing that numbers were less than 500 per gram of air-dried hay.

Since *S. bovis* could grow on the ingredients of the feed without added rumen fluid, its ability to produce nutrients for the cellulolytic cocci requiring rumen fluid was tested. Four grams of poor quality hay and 1 g. of dairy feed concentrate\* were boiled with 140 ml. of inorganic culture solution and filtered. The filtrate was divided into two equal portions which were sterilized and cooled. One was buffered by adding 0.3% sodium carbonate and bubbling with carbon dioxide and was inoculated; the other served as a control. After incubation at 38° C. the inoculated culture showed typical development. At 48 hours the flasks were removed and to each were added 1.2 g. of agar and 30 ml. of a suspension of finely divided cellulose, and the contents were resterilized. Experimental and control tubes were brought to the same pH and inoculated with four strains of cellulolytic cocci isolated from the rumen. The strains were also inoculated into the usual rumen fluid cellulose agar medium (9).

The growth of *S. bovis* exerted no favorable influence on the development of any of the cocci tested. Coccal strains 43-1 and 46-1 grew only in the rumen fluid medium. Strains 61 and 69 showed about the same development in the three different media.

The actively amylolytic coccus described by van der Wath (17) possessed many of the characteristics of *S. bovis*. In addition to attacking starch, lactose, inulin, raffinose, arabinose, and glucose, it survived a temperature of 60° C. for 30 minutes. Sorbitol and mannitol were not fermented. It differed from *S. bovis* in failing to ferment salicin and in being strongly iodophilic.

A strain of *S. bovis* which had been carried for some time in the laboratory was inoculated into both liquid and agar media containing starch and the resulting cells tested for iodophily. None was found, even though the test was made 6 to 8 hours after inoculation when growth was rapid. A culture freshly isolated from a sheep (Pullman) was similarly tested but no iodophily found. Of 25 strains of rumen streptococci tested by Hobson and Mann (7) only seven showed iodophily and these only when the cells were old. The coccus of van der Wath, although similar in many respects to the tested strains of *S. bovis*, is apparently not identical with them. Whether it represents a different species or a variant cannot be determined until additional

\*Forty parts corn gluten feed, 54 hominy and cornmeal, 38 distillers' dried grains, 36 brewers' dried grains, 8 linseed oil meal, and 20 molasses.

strains of amylolytic cocci have been isolated from the rumen of animals in widely separated geographic localities and their characteristics described.

The streptococci isolated from sheep by MacPherson (13) are quite similar to the *S. bovis* strains except that glycerol was occasionally fermented and mannitol was not. The aerobic plating technique used would select against organisms as anaerobic as those of the present study and may explain the fermentation differences.

### The Kinds of Cellulolytic Bacteria Observed

The cellulolytic colonies were identified by inspection of the colony itself and by phase microscopy of fresh mounts. When several very similar colonies were in the same tube, usually only one was examined microscopically. Most of the cellulose digesters could be classified into one of the following groups: (1) cocci 0.8–1.2  $\mu$  in diameter, single or diplo, or in chains, forming discrete colonies; (2) small rods similar to *Bacteroides succinogenes*, forming no discrete colony and moving through the agar as the cellulose dissolved; (3) curved rods, often motile, forming discrete colonies with usually only a narrow zone of cellulose digestion, similar to the butyric rod previously reported (9); (4) large sporeforming rods, non-motile, forming a diffuse irregular colony; and (5) minute cocci 0.3–0.4  $\mu$  in diameter. The occurrence of these types in the initial rumen fluid cellulose agar cultures is shown in Table V. Table VI summarizes their incidence.

Many of the colonies, which could not be identified by macroscopic and microscopic examination, were subcultured to dilution tubes of rumen fluid cellulose agar, because they might be new types. In a few instances the subcultures failed to grow, but in most cases as they were freed of accompanying bacteria they could be assigned to one of the above groups. Many of the unidentified colonies would probably also have fallen into one of these groups but they could not be cultured.

As seen in Table VI, the butyric rods occurred most commonly, followed by the cocci, sporeformers, *Bacteroides succinogenes*, and the tiny cocci. The sporeformers were detected in most animals, followed by the cocci and the butyric rods. The cocci, the butyric rods, and *B. succinogenes* had been identified as important digesters of cellulose in the rumen, but the sporeformers and tiny cocci had not previously been encountered. Only a few colonies of the tiny cocci were seen and none grew in subculture. Representatives of the other types were isolated and, together with strains from other sources, were studied in more detail. The results for the sporeformers and the cellulolytic cocci are reported.

### Sporeformers from the Rumen

The rather large numbers of cellulolytic sporeformers (Tables V and VI) in the rumen contents of the animals fed timothy hay, and the regularity of their occurrence indicated an importance in the rumen. Three strains from animals 28, 52ELO, and 66, respectively, were isolated and their character-

TABLE V  
NUMBERS AND KINDS OF CELLULOLYTIC BACTERIA IDENTIFIED  
(millions per milliliter)

		June				July				August				September			
		Ration	Sporiformers	Cocci	Bacteroides	Butyric rods	Sporiformers	Cocci	Bacteroides	Butyric rods	Sporiformers	Cocci	Bacteroides	Butyric rods	Sporiformers	Cocci	Bacteroides
Animal	3	7	2					2		4	0.06	2		0.2	0.06	0.2	
18	4	4	0.08					4		0.2	2	2			0.04		
24	6	4						4			0.2						
28	8	4															
35	1	4	0.2		0.02												
42WW	3	2		0.04		0.4	0.08	10		4	0.08			4	0.04		0.6
42OK	4	2				0.14	0.02			2	0.02			0.8	0.002		0.2
43	7			0.04		0.04	2	2		0.8	2	2		0.08			0.06
44	8			0.4			0.04	0.4		2	0.04	0.4		2	0.002		0.02
45	2			0.2	0.02	48	2	2		2	2	2		2	0.002		0.08
51	6		2				0.02				0.14			10	0.002		
52	1							0.04			0.08			2	0.28		0.2
52ELO	2			0.2				4			2		0.1	2			0.4
52WW	2		2					4		4	2	6					0.016
55	5										0.2						0.4
56	5		2								0.2						
57	5							0.006			0.2	6		2			
58	8							0.16			0.04	6					
59	3			0.04				0.04			0.04		0.34		0.002		0.2
61	5			0.2				2			0.04			0.2	0.2	0.06	
66	8		0.02					4		0.4	0.04			0.2	0.04		
67	8		0.2					2	0.02	2	0.2	2		8	0.003		0.04
68	7		0.12		0.004			2		2	0.2	2					
69	4		0.2	0.2			0.1	2		2	0.28						
Average			0.618	0.137		2	0.013	1.3		0.7	0.359	1.5	0.02	1.27	0.031	0.01	0.092



TABLE VI  
OCCURRENCE OF CELLULOLYTIC TYPES IN THE COWS FED TIMOTHY HAY

	Per cent of the cellulolytic colonies					Per cent of the animals in which found				
	June	July	August	Sept.	Total	June	July	August	Sept.	Total
Sporeformers	21	5	0.7	14	6.8	60	12	100	50	54
Cocci	4	52	29	5	26.8	30	83	39	9	41
<i>Bacteroides</i>	0.02	0.03	0.4	4	0.3	10	4	9	9	8
Butyric rods	66	32	26	39	39.5	10	38	43	50	35
Tiny cocci	0.03	0	0	4	0.1	10	0	0	9	4
Unidentified	9	11	44	34	26.5					

istics studied. The remaining strains from the cows fed timothy hay resembled these three morphologically. Additional strains were encountered when a tablet of dried rumen bacteria prepared by a veterinary supply house was examined for cellulolytic bacteria. None of the previously encountered non-sporeforming rods and cocci were found but several colonies of sporeformers appeared in the 600X dilution in rumen fluid cellulose agar. Five of these were pure cultured and designated B1 to B5. During studies in Mississippi (11) another strain (No. 30) was found when rumen contents from an animal on poor grass pasture were diluted into rumen fluid cellulose agar tubes. No sporeformers were encountered in cultures inoculated from the animals on Ladino clover pasture.

The deep colonies of these sporeformers in rumen fluid cellulose agar were small, irregular in outline, not very opaque, and often with a seemingly empty center. Surface colonies in cellulose agar were almost invisible except for the zone of cellulose digestion. They spread rapidly as a thin layer with fingerlike extensions which could be detected under the dissecting microscope if the light was carefully adjusted. Clear spots of cellulose digestion were usually visible in rumen fluid cellulose agar 24 hours after inoculation and all cellulose in the tube was often digested within a few days. A very considerable gas pressure developed.

The oval to oblong spores formed at one end of the sporangium. The sporangia were of the clostridial type (Fig. 1), spindle-shaped, and distinctly different in shape and size from the vegetative cells, which were non-motile rods. The clostridia occurred in large masses in certain parts of the colonies in rumen cellulose agar. In some preparations they were attached by their tips in rosettes of three to six cells (Fig. 2). The distal tips were in turn attached to other cells, giving a striking three-dimensional network. The appearance suggested that the clostridial forms divided. Spores developed in the clostridia as shown in Fig. 1. In most of the strains the sporangial wall initially remained attached to the spore but after culture in the laboratory for a long time this characteristic was often absent. Strain 30 never showed the old sporangial wall attached, even when first isolated. This strain differed also in forming small amounts of yellow pigment.

Spores were observed in those parts of the colony just inside the periphery. As the colony enlarged a shell of spores was always found in the distal portion and those more centrally located disintegrated. Also in the long fingerlike surface growths the spores were found near the tips but were almost entirely absent from the older portions. In many old cultures no spores at all were found in the areas cleared of cellulose. They formed initially and then completely disappeared. This behavior was somewhat variable. Spores were usually formed more abundantly on first isolation and there was some indication that the abundance of spores was influenced by the particular batch of culture medium. Many of the strains were lost because subcultures failed to grow.

The heat resistance of the spores was not determined, though strain 30 was viable after being heated to 80° C. for 1 minute. During the initial isolations subcultures often failed to grow unless fairly massive inocula were used. Also, the number of colonies developing was usually less than expected. Preliminary tests suggested that the organisms might be sensitive to oxygen. A colony was divided in two, and one half subcultured with as little exposure to air as possible. The other half was held for 5 minutes on the end of the microspatula used for inoculating and then similarly subcultured. The inoculum protected from air gave many more colonies though a few developed from the one exposed. In another experiment, exposure to air for 2 minutes significantly decreased the viability, though not to the same extent. These results suggest that oxygen kills the cells though it is possible instead that their growth was inhibited by the small amount of oxygen added with the inoculum. The sporeformer strains were extremely sensitive to oxygen and never showed growth if resazurin was oxidized. In some cases, even though this indicator reduced, it was suspected that failure to grow was due to too high an oxygen tension in the medium.

The sporeformers do not require rumen fluid. They grow on many common laboratory media and show a rapid development in an infusion of hay plus concentrate. Their ability to digest alfalfa hay was tested by inoculating into rumen fluid broth in tubes containing 40 mg. air-dried hay. Uninoculated control tubes incubated in parallel contained 22.7 mg. dried material. The tubes inoculated with strains B4, B5, 30, 66, and 52ELO showed weight decreases of 44, 45, 47, 32, and 50%, respectively. The residues were not further analyzed to measure the digestion of the various constituents of the hay but the decrease in total weight suggests that more than one fraction was attacked (see later section on hay digestion by the cocci). Strains 66 and 52ELO caused decreases of 22 and 41% in the residual weight of poor quality grass hay. These results support the view that the sporeformers play a part in fiber digestion in the rumen.

Other characteristics of the sporeformers were as follows: strain 30 gave no visible growth in beef extract-peptone broth or in brain heart infusion broth; no visible growth in nutrient gelatin but the gelatin completely liquefied in 96 hours; no visible growth in MR-VP broth, and the methyl

PLATE I

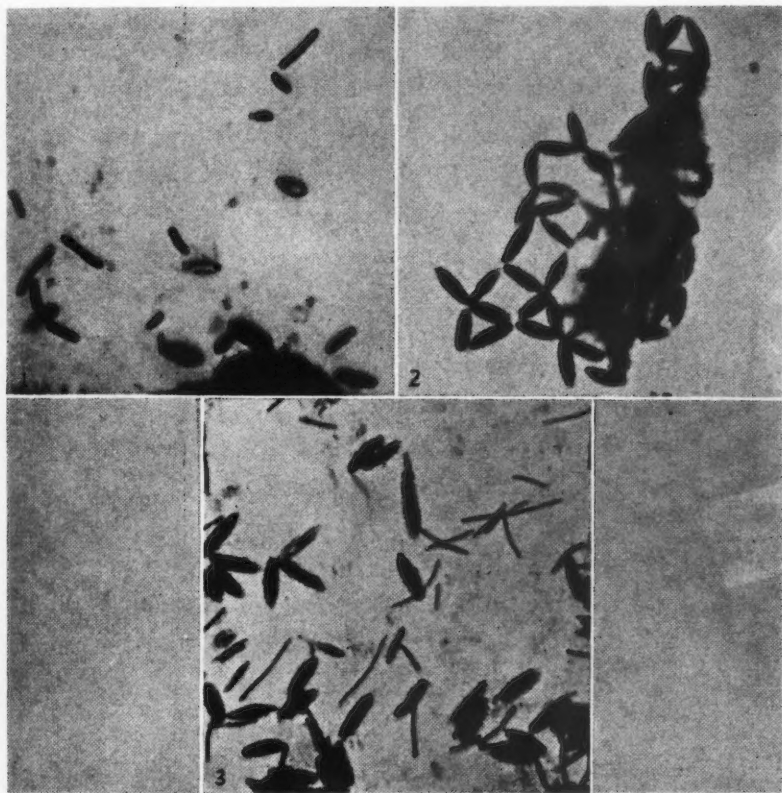


FIG. 1. Gram stain of *C. lochheadii*, strain 30, from a 24-hour rumen fluid glucose agar culture. Vegetative cells and a few larger cells regarded as clostridia. Mag. 1550 $\times$ . Green filter.

FIG. 2. Same smear as Fig. 1. Selected portion to show clostridia and their arrangement.

FIG. 3. Smear of 48-hour culture of *C. longisporum* in rumen fluid cellobiose agar. Vegetative cells and clostridia. Gram stain. Mag. 1550 $\times$ .

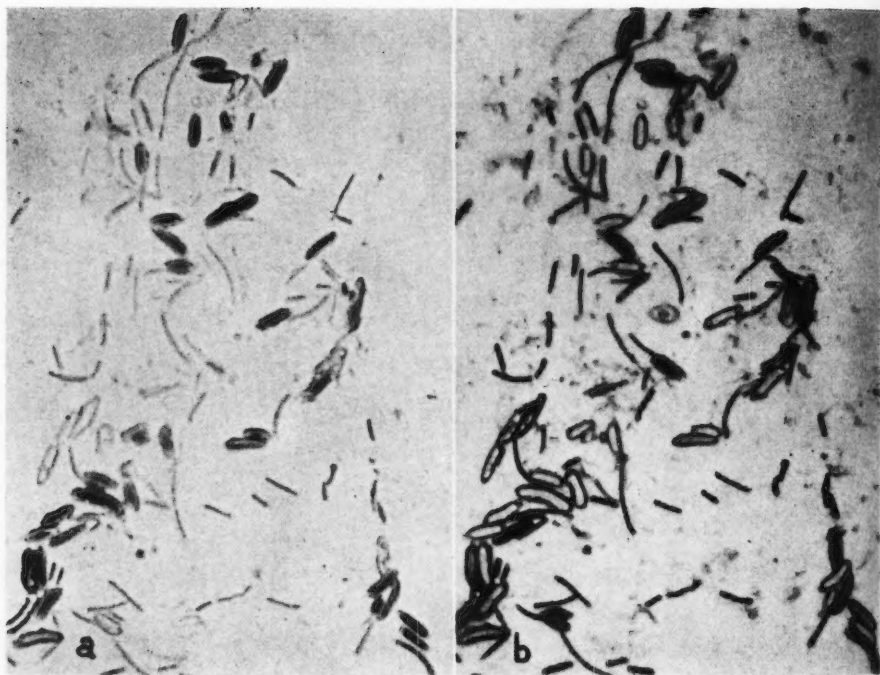


FIG. 4. Smear of 5-day culture of *C. longisporum* in rumen fluid cellulose agar. Vegetative cells and clostridia containing spores. Spore stain with malachite green and safranin counter stain. (a) Red filter. Spores show as dark areas in center of clostridia. (b) Green filter. Vegetative cells distinct. Spores almost colorless.

red and Voges-Proskauer tests both negative; acid and gas in Kligler's iron agar, no  $H_2S$ ; litmus milk reduced in 24 hours and completely peptonized in 48 hours; gas and acid in thioglycolate agar. Strains B4 and B5 showed similar characteristics except that gelatin was not liquefied by B5 until after 20 days and by B4 only partially after 20 days.

Substrates fermented by strains 28, 52ELO, 66, and B1 to B5 are shown in Table VII. Of the sugars commonly attacked only two were monosaccharides. These tests were made with a medium containing rumen fluid and 0.4% substrate. Copious slime was produced in the tubes containing fermentable sugars. In some, the extracellular slime was so abundant that the medium gelled and the tube could be inverted without flow of the contents. Substrates not fermented were D-xylose, L-arabinose, D-arabinose, galactose, mannose, rhamnose, trehalose, lactose, raffinose, inulin, and esculin.

TABLE VII  
SUBSTRATES FERMENTED BY THE SPOREFORMERS

Substrate	Strain								
	28	52ELO	66	B1	B2	B3	B4	B5	30
L-Xylose	-	-	+		-		-	-	
Glucose	+	+	+	+	+	+	+	+	+
Levulose	+	+	+	-	-	-	-	+	
Cellobiose	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	
Sucrose	+	+	+	+	+	+	+	+	
Starch	+	+	+						
Salicin				+	+	+	+	+	
Cellulose	+	+	+	+	+	+	+	+	+

The fermentation products of strain 52ELO were determined. It was grown in all-glass sealed flasks with 1 g. of poor quality grass hay and 0.5 g. concentrate as substrate. The weight of insoluble substrate digested was 335 mg. but since soluble substrates were also present this cannot be used as the basis for calculating carbon recovery. Products formed, in mM., were: carbon dioxide 2.55, hydrogen 2.76, acetic acid 0.92, formic acid 0.52, butyric acid 0.87, and ethanol 0.54. No succinic acid was formed and only a trace of lactic acid. The large proportions of hydrogen and carbon dioxide account for the considerable gas pressures observed in the culture tubes.

The characteristics of these sporeformers differ in several respects from the previously described species of *Clostridium* and necessitate that they be assigned to a new species. In recognition of Dr. Lochhead's pioneer work in demonstrating cellulose digestion by colonies of aerobic bacteria (12) and in further recognition of his important contributions to our knowledge of bacteria as they exist in nature this new species is named *Clostridium lochheadii* with the following characteristics.

Gram-positive rods 0.7–1.5  $\mu$  by 3–7  $\mu$ . Non-motile. Anaerobic. Form oval to oblong spores 1–1.5  $\mu$  by 2–3  $\mu$ . Spindle-shaped clostridia, 1.5–1.7  $\mu$  by 5–6  $\mu$  with tapered ends, are formed prior to sporulation and the spores develop terminally and slightly laterally in them. The clostridia are often arranged in star-shaped aggregates, with the cells joined by the tips and these aggregates in turn joined to form a three-dimensional network. The appearance suggests that the clostridia divide. Spores form in the peripheral part of a colony, degenerate, and are replaced by new ones formed as the colony grows out. Part of the sporangial wall often remains attached to the spore, extending from one end as a tube. Spores are unstained by simple stains but show the typical reaction to spore stains.

Subsurface colonies are irregular in shape in cellulose agar, somewhat diffuse, and the center usually less dense than the periphery. Colonies spread rapidly on the surface of cellulose agar.

Cellulose is rapidly fermented and also glucose, cellobiose, maltose, sucrose, starch, and salicin. Levulose often and L-xylose seldom fermented. Not fermented were D-xylose, L-arabinose, D-arabinose, galactose, mannose, rhamnose, trehalose, lactose, raffinose, inulin, and esculin. Forty to fifty per cent of the insoluble materials in alfalfa hay were digested. Copious slime formed when carbohydrates were attacked.

Growth occurs on a wide variety of media containing fermentable carbohydrate, including infusions of various ruminant feeds. Does not require rumen fluid. No visible growth in beef extract – peptone broth or beef heart infusion broth. No visible growth in nutrient gelatin but gelatin liquefaction may be complete within 96 hours, or only partial. No visible growth in MR-VP broth and the methyl red and Voges-Proskauer tests both negative. Acid and gas in Kligler's iron agar; no  $H_2S$ . Litmus milk reduced in 24 hours and completely peptonized in 48 hours. Gas and acid in thioglycolate agar.

Habitat: the rumen of cattle.

Another sporeformer was first observed as a very orange colony in the rumen fluid cellulose agar<sup>1</sup> tube (200,000 $\times$  dilution) inoculated from animal 52WW. It produced much more pigment than any of the other cellulolytic bacteria thus far observed. The vegetative cells were motile rods 1  $\mu$  in diameter by 7–15  $\mu$  long. Some of these transformed into clostridia 2–3  $\mu$  by 7  $\mu$  (Fig. 3) in some of which slightly curved spores 1  $\mu$  in diameter and 3–6  $\mu$  long were seen (Fig. 4). The curvature of some of the long spores appeared to be slightly spiral rather than in a flat plane.

Strain 52WW was extremely difficult to maintain in subcultures. Large inocula often failed to grow and even when growth occurred only a few colonies formed. A pure culture was obtained by picking from rumen fluid cellobiose agar and was maintained for about 3 months before it was lost by too long a delay in subculturing. The spores resemble those of *C. lochheadii* in losing their viability within a few weeks.

The carbohydrates fermented were cellulose, dextrose, galactose, cellobiose, and sucrose. Much slime was produced on these sugars. Cellulose digestion was rapid but not as rapid as by *C. lochheadii*.

The strain grew on nutrient broth or yeast extract if cellulose was present. Growth with carbon dioxide and bicarbonate was better than with nitrogen gas.

Fermentation products from 1.14 mM. cellulose (as hexose) were, in mM.: hydrogen 0.59, carbon dioxide 1.13, acetic acid 0.51, formic acid 0.75, and ethanol 0.26. No propionic, butyric, lactic, and succinic acids were produced.

Strain 52WW differed from *C. lochheadii* in being motile, in producing very long spores, much more yellow-orange pigment, no butyric acid, and in



fermenting galactose. These features necessitate placing it in a separate species, *Clostridium longisporum* n. sp., with characteristics as described above.

The habitat of *C. longisporum* is unknown. In the dilution series in which it was originally isolated there were no yellow-orange colonies visible in the next lower dilution so it may have been a contaminant. Once previously an organism similar in color and cellulolytic activity was found as an undoubted contaminant in a rumen cellulose agar series, but was lost before other characteristics could be ascertained.

### The Cellulolytic Cocci

Seven strains isolated from the cows on the timothy hay rations were picked because they represented the range of differences in colony types. Other strains were isolated from sheep at Ithaca. Some of the characteristics are shown in Table VIII.

The reaction of the rumen cellulolytic cocci to the Gram stain is not uniform. The most thorough study of the Gram reaction was made with strain 69. An 8-hour culture in rumen cellobiose broth was subcultured to a tube of similar medium, which was incubated 12 hours, and the cells then Gram stained in parallel with *Escherichia coli* and *Streptococcus pyogenes*. The cells were Gram-negative without exception. The slightly more intense color of the cocci as compared with *E. coli* was just as marked when safranin was applied as a simple stain as when it was part of the Gram procedure. With the other strains, the Gram reaction was often variable.

Strain S2-23 was observed in the first dilution tube of a rumen fluid cellulose agar series inoculated with rumen contents from a sheep which had been given excess glucose 10 hours prior to sampling. The culture count of cellulolytic bacteria before the glucose was administered was 10 million per milliliter, most of the colonies appearing to be the non-sporeforming butyric rods. Following the administration of glucose the rumen contents became quite acid (10) and the animal died 7 days later. Ten hours after the glucose was given another sample of rumen contents was removed and diluted into a rumen cellulose agar series. These showed 500 cellulolytic colonies per milliliter of rumen contents, a great decrease from the earlier count. Colonies of Gram-positive streptococci were prominent among the cellulolytic colonies from the acid rumen and one of these was isolated in pure culture as strain S2-23. Out of 24 colonies examined from the pre-glucose series none were of this type, indicating that they were much outnumbered by other cellulose decomposers in the original rumen contents but were relatively more abundant in the acid rumen. Smears of the rumen contents before and after administration of the glucose were examined and streptococci identical in morphology with the pure culture were observed in both, but were much more numerous in the normal contents, indicating that the streptococci also diminished in numbers with acid but not as rapidly as the other forms. Strain S2-23

TABLE VIII  
CHARACTERISTICS OF SOME OF THE RUMEN COCCI

No. of strain	Date of isolation	Source	Ration	No. per ml.	Size ( $\mu$ )	Arrangement	Gram reaction	Capsule	Colony color
43-1	8-3-51	Cow 43	No. 7	$2 \times 10^8$	1-2	Single to short chains, many diplo	G-	Present	Rarely yellow
46-1	7-5-51	Cow 46	No. 6	$2 \times 10^8$	0.7-1.3	Single to short chains			White
50-2	7-5-51	Cow 56	No. 2	$4 \times 10^8$	0.7-1.1	Short to very long chains	G-	Slight	Rarely yellow
61	6-15-51	Cow 61	No. 3	$2 \times 10^8$	1-1.5	Single and diplo			White
61-1	7-5-51	Cow 61	No. 3	$2 \times 10^8$	0.7-0.8	Single to short chains	G+	Present	Yellow
66-1	9-7-51	Cow 66	No. 5	$1 \times 10^8$	0.8-3.0	Single to short chains	G-	Present	Yellow
69	6-15-51	Cow 69	No. 4	$6 \times 10^8$	0.6-2.0	Single or diplo	G-	Copious	White
S1-2	8-22-50	Sheep 1	Alfalfa hay	$6 \times 10^8$	1.0-1.2	Single or diplo	G+		White
S1-y	8-22-50	Sheep 1	Alfalfa hay	$3 \times 10^8$		Single or diplo	G+		Yellow
S2-23	10-11-50	Sheep 2	Alfalfa hay + excess glucose	10	0.8-0.9	Single to long chains	G+		Slightly yellow

digested cellulose much more rapidly and completely than the strains of streptococci previously obtained. Unfortunately, the culture was lost before the fermentation products could be analyzed.

The sugars fermented by 10 strains of streptococci are shown in Table IX. None of the tested cocci fermented glucose, L-xylose, D-xylose, D-arabinose, galactose, rhamnose, raffinose, inulin, trehalose, esculin, salicin, and starch. Cellobiose was the only sugar fermented by all strains.

TABLE IX  
SUGARS FERMENTED BY THE COCCAL STRAINS

	46-1	56-2	61	61-1	66-1	69	S1-2	S1-y	S2-23
L-Arabinose	±	+	-	-	-	-	-	-	-
Cellobiose	+	+	+	+	+	+	+	+	+
Lactose	±	-	-	-	+	±	+	-	+
Maltose	±	-	-	-	-	±	-	-	-
Mannose	+	-	-	-	±	-	-	-	-
Sucrose	±	-	-	±	-	+	-	-	-
Fructose	-	+	-	-	-	-	-	-	-

The results of the analyses for fermentation products are shown in Table X, together with the fermentation products of other cocci reported in the literature. The values for carbon dioxide involved a considerable error owing to use of 100% carbon dioxide and 0.5% sodium bicarbonate in the medium. The equilibration between gas and liquid phases was not always complete.

According to their fermentation pattern certain of the cocci are closely related, but many show differences indicating diverse origins. Types producing much or little of a certain product can be designated, but there are intergradations of types, and strain S1-2 produced at least a small quantity of each product.

The products shown in Table X can arise from one fermentation pattern, e.g. that of *Escherichia coli*, and there is no evidence to exclude the possibility that the differences in metabolic products of the cellulolytic cocci are due to variations in a basic pattern common to all strains. On this basis they should be included in a single genus.

The first generic designation for a member of this group was *Ruminococcus*, with *R. flavefaciens* as the type species (15). In order to accommodate all the strains of cellulolytic cocci in the genus it is necessary to redefine it as follows: *Ruminococcus* (15)—Spherical cells, somewhat elongated prior to division; single, in twos or in chains, never in tetrads or cubical packets. Non-motile, non-sporeforming. Gram-positive or -negative or -variable. Anaerobic. Ferment carbohydrate to form acetic acid, at least traces of hydrogen, and various combinations of ethanol, formic acid, lactic acid, and succinic acid. Type species *Ruminococcus flavefaciens* Sijpesteijn.

TABLE X  
PRODUCTS OF FERMENTATION BY THE COCCI

Strain	Substrate		Products formed, mM. hexose fermented							Carbon recovery, %	
	Source	Kind	Amount fermented, mM.	H <sub>2</sub>	CO <sub>2</sub>	Ethanol	Acetic acid	Formic acid	Lactic acid		Succinic acid
61-1 <i>Ruminococcus flavefaciens</i> S	Cow	Cellulose	0.83	0.037			0.5	0.193	0.094	0.675	70
52 Maki (14)	Sheep	Cellulose	1.6			None	0.65	0.64	Trace	0.51	66
SI-y	Sheep	Cellobiose	2.05			None	0.73	0.58	—	0.5	67
F-6 Hall (5)	Cow	Cellulose	1.78			None	0.15	0.1	0.26	1.03	88
SI-2	Sheep	Cellulose	0.32	0.04	-0.882	None	0.32	0.06	0.4	0.69	78
43-1	Rabbit	Cellulose	0.58	0.63	1.65	0.47	0.47	0.655	Trace	0.378	82
69		Cellulose	0.58			0.48	0.22	Trace	None	0.175	70
Colorless coccus U		Cellulose	0.59			0.39	0.29	Trace		0.27	
Hungate (9)	Sheep	Cellulose	2.71	Trace	0.436	0.606	0.362	0.48	0.213	1.06	65
SI-y	Cow	Cellulose	0.356	0.99		0.52	0.68	—	0.43	None	62
Yellow coccus A		Cellulose	<0.58			>0.267	>0.224	>0.038	None	None	
66-1	Cow	Cellulose	0.85	1.15	1.79	0.667	0.628	0.035	0.07	None	77
SI-y	Steer	Cellulose	1.08	0.224	0.466	0.476	1.08	0.25	0.10	None	69
66-2	Cow	Cellobiose	1.17	0.04	0.1	0.21	0.61	0.25	0.61	None	64
SI-y	Cow	Cellulose	0.475	0.417	0.198	0.559	0.810	None	1.022	None	100
SI-y	Steer	Cellulose	1.13	0.233	0.27	0.223	0.486		0.6	Trace	75
Hungate (9)	Cow	Cellulose	0.35	0.13		Trace	-0.19		0.384	None	26
46-1		Cellobiose	0.58		0.148	0.102	0.029	0.147	0.378	None	26
46-1	Cow	Cellulose	1.09	0.124		0.14	0.38	0.141	0.403	None	42

On the assumption that strain 61-1 of Table IX is identical with Sijpesteijn's strain S (the only one for which fermentation products were determined), production of hydrogen should be added to the characteristics of *R. flavefaciens*. The yellow color is not always exhibited, though usually present under certain conditions. To this species may be assigned also strains S1-y, F-6, S1-2, and probably 52 and 53 of Table X.

Strains 69, 56-2, 46-1, 43-1, and U (9) are considered to belong to a new species, *Ruminococcus albus*, which characteristically produces none or little of the yellow pigment. The colonies are usually white. Deep colonies are initially lens-shaped, but soon become multiple lens-shaped and often spread out in a complex arrangement. Cells usually single or in twos, often slightly elongated prior to division, 0.8 to 2.0  $\mu$  in diameter, Gram-negative to Gram-variable. Capsule often formed. Ferments cellulose and cellobiose, but usually does not ferment glucose and other sugars. Nutrient requirements are not met by usual bacteriological culture media, but can be met by rumen fluid, extracts of feces, or other media containing metabolic products of other microorganisms. Hydrogen, carbon dioxide, ethanol, acetic acid, formic acid, and lactic acid are produced in various combinations and proportions, but no succinic acid. Usually produces more hydrogen and carbon dioxide than does *R. flavefaciens*. Usually not iodophilic. Strain 69 is the type strain. Habitat: rumen of cattle and sheep.

The yellow coccus strain A is considered a variant of *R. flavefaciens*, producing less than the usual amount of succinic acid. Strain 66-1 does not easily fit either of these species. The low carbon recovery suggests that not all the fermentation products were identified.

The number of cellulolytic cocci in the rumen is one measure of their importance. Bryant and Burkey (2) found that they comprised 5 to 10% of the total culture count. A second measure is the degree to which they can digest the insoluble constituents of roughages. In measuring this ability it is important that growth of the test organism be limited only by the amount of added roughage. With a low concentration this requirement is fulfilled and the concentration of metabolic products remains below the inhibitory level.

Air-dried ground alfalfa hay (40 mg.) was weighed into each of numerous culture tubes containing 10 ml. of 30% rumen fluid broth. These were inoculated with the test cultures and incubated 2 weeks at 39° C. The supernatant fluid was removed, the sediment suspended in a little distilled water and transferred to a weighing tube. The plant fiber particles soon settled and the supernate with its still-suspended smaller particles (including the bacteria) was again removed. This washing process was repeated until the supernate was clear. The sediment was dried and weighed. Uninoculated tubes similarly treated showed the dry weight of sedimented fiber particles in the alfalfa to be 22.7 mg. The percentages of the insoluble materials digested by the tested strains were: 21 for 61-1, 35 for 43-1, 33 for 66-1, and 45 for 69.

The utilization of various fractions of hay by other coccal strains was tested. The experiment was similar to the above except that after determination of

the residual dry weight the residue was successively treated with benzene-alcohol, hot water at 85° C. for 24 hours, 2% (w/v) sulphuric acid at room temperature for 24 hours, and 70% (w/v) sulphuric acid at room temperature for 24 hours. After each treatment the fiber was washed, dried, and weighed. The amount of each fraction digested was calculated and expressed as a percentage of the total weight of that fraction. The results are shown in Table XI for two different feeds, alfalfa hay and a poor quality grass hay.

Between 44 and 59% of the materials in the alfalfa hay were digested and 19 to 29% of the grass hay. The action on the benzene-alcohol fraction was about the same for the two hay samples. The amount of this fraction was small. The hot-water-soluble fraction in the alfalfa was digested to a greater extent than in the grass. Fractions 3, 4, and 5, regarded as consisting chiefly of hemicellulose, cellulose, and lignin, respectively, showed more digestion in the alfalfa than in the grass. The digestion of the "lignin" fraction in the alfalfa was unexpectedly high and was of significant magnitude even with the grass hay.

All of the fractions of the alfalfa hay were attacked to about the same extent. With the grass hay, the attack on the "cellulose" fraction was usually greater than that on any other except the benzene-alcohol. Although the method for estimating these different fractions was crude, the results indicate that susceptibility to acid hydrolysis is not closely correlated with digestibility by the bacteria.

### Discussion

During the past 10 years various cocci and non-sporeforming rods of the rumen have repeatedly been identified as cellulose digesters. The present study confirms their importance and provides additional information on their characteristics. The difference in succinic acid production by strain S of Sijpesteijn (15) and the yellow cocci (9) is apparently not due to differences in the culture conditions. The bacteria themselves differ. Also other variations in the characteristics of individual strains show that the rumen cellulolytic cocci are not a homogeneous group. Study of additional strains may disclose better defined and more restricted species than *R. flavefaciens* and *R. albus* but present information is insufficient to warrant further splitting. Cellulose digestion is not regarded as a distinctive characteristic of the rumen cocci. So much variation has been observed in the rate of cellulose digestion by pure cultures that it seems highly probable that non-cellulolytic strains referable to *R. flavefaciens* or *R. albus* exist in the rumen.

The true relative importance of *Bacteroides succinogenes* in the rumen is probably not disclosed by the present experiments. Many strains apparently do not grow readily in cellulose agar (3), though cellulose is rapidly digested in broth tubes. Indications of sporadic cellulose digestion by *B. succinogenes* were observed when it was first isolated (8) and repeatedly since that time. The factors concerned have not been identified, but they almost certainly affected the agar dilution experiments of the present study and the counts of *B. succinogenes* do not reflect its relative importance.



TABLE XI  
DIGESTION BY COCCAL STRAINS OF VARIOUS FRACTIONS OF ALFALFA HAY AND POOR GRASS HAY

Fraction No.	Description	Alfalfa hay				Poor quality grass hay			
		Per cent digested				Per cent digested			
		Control, mg.	Colorless coccus	Sl-y	Sl-2 S2-23	Control, mg.	Colorless coccus	Sl-y S1-2	S2-23
1	Soluble in benzene-alcohol	1.0	30	50	30	0.9	56	45	33
2	Soluble in H <sub>2</sub> O, 85° C.	2.2	73	46	64	1.3	15	15	31
3	Soluble in 2% H <sub>2</sub> SO <sub>4</sub>	8.3	49	47	51	6.8	15	29	18
4	Soluble in 70% H <sub>2</sub> SO <sub>4</sub>	8.1	36	62	53	10.9	23	31	27
5	Insoluble residue	3.7	38	16	49	3.6	6	19	17
Total dry weight		23.3	44	54	53	23.5	19	29	23
					59				25

On a number of occasions the low dilution tubes of cellulose agar series inoculated with rumen contents have shown an extremely rapid and complete disappearance of all the cellulose. In the next lower dilutions, zones of cellulose digestion developed only after much longer incubation, indicating that the inoculum contained only a few colony-producing units of the rapid cellulose digester. Of all the isolated cellulolytic rumen bacteria, *C. lochheadii* is the only one which shows such rapid growth from a few initial cells. In deep agar colonies its zone of cellulose digestion increases only slightly more rapidly than with most other cellulolytic bacteria, but in the thin agar of roll tubes the cells soon reach the surface and then spread vastly more rapidly than other forms thus far encountered. If the rapid and complete cellulose digestion be ascribed to *C. lochheadii*, this organism is fairly widespread in the bovine rumen though often in small numbers.

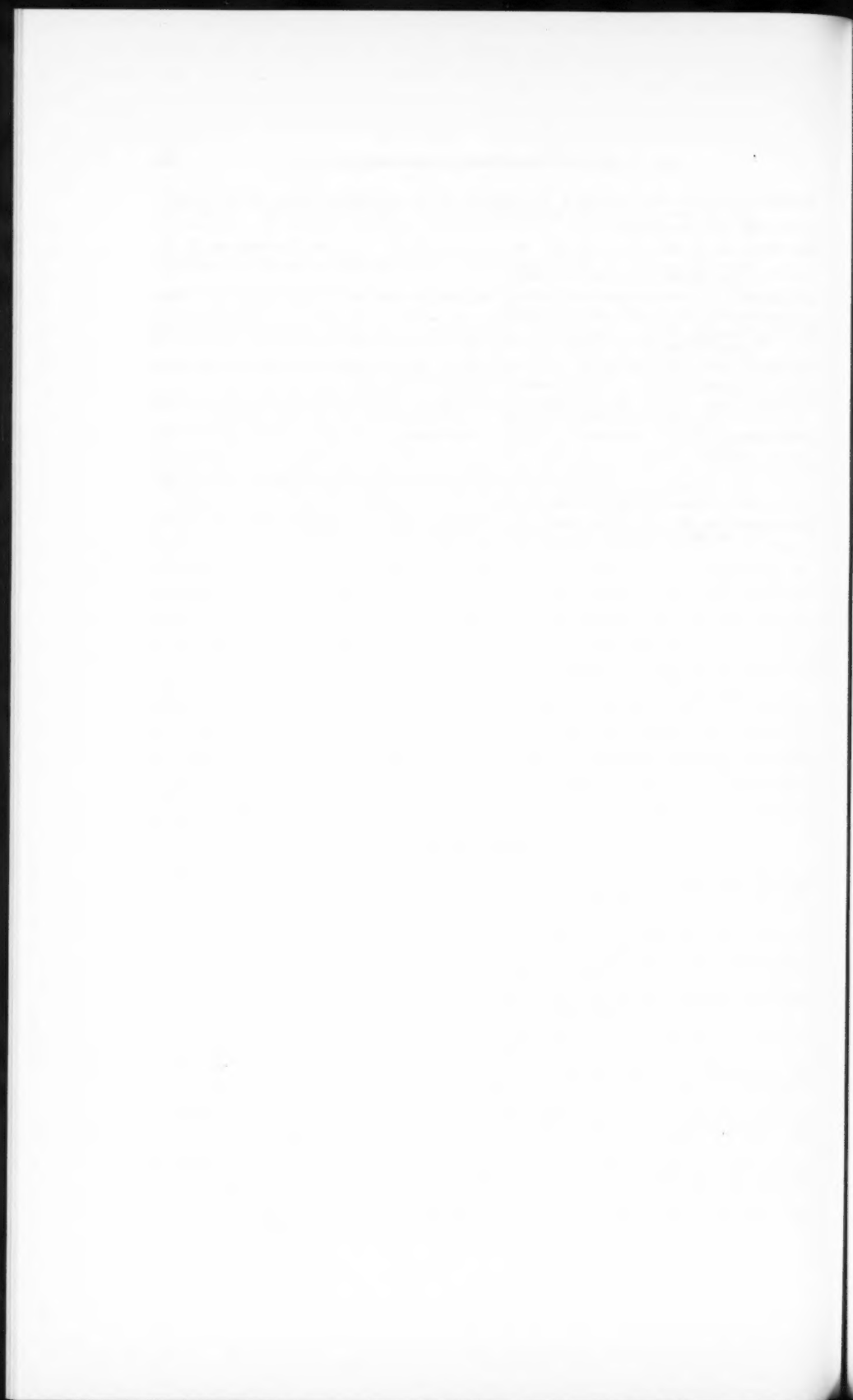
When a colony of *C. lochheadii* in cellulose agar is subcultured, the number of colonies developing is much fewer than when other strains of cellulose digesters, showing equal clearing of cellulose, are transferred. The numbers demonstrable by culturing are relatively less than the cellulose digestion or, conversely, the cellulose digestion by this species is greater than the numbers indicate. If this observation with agar cultures applies also to the rumen, *C. lochheadii* is of considerable importance with certain rations.

The minute cocci seen in areas of cellulose digestion during the present experiments suggest that they represent an additional cellulolytic type. However, the capacity to digest cellulose should not be inferred until proved with pure cultures. It will be interesting to see whether these cocci prove to be cellulolytic and also whether additional kinds of cellulose-digesting bacteria of the rumen will be discovered. Whenever ruminants are fed a distinctive type of roughage it would seem profitable to determine the kinds of cellulolytic bacteria concerned.

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## THE INHIBITION OF KETO ACID OXIDATION BY PYOCYANINE<sup>1</sup>

J. J. R. CAMPBELL, A. M. MACQUILLAN, B. A. EAGLES, AND R. A. SMITH<sup>2</sup>

### Abstract

When tested against *Pseudomonas fluorescens*, pyocyanine was found to stop the oxidation of a number of substrates at the keto acid level. This inhibition could be reversed by the addition of divalent cations. Of these, magnesium was most effective. The pigment was found to be similarly effective against the oxidations of *Proteus vulgaris*. Whole cells of *Escherichia coli* were not affected by the dye, whereas cell extracts were, indicating that the dye did not penetrate the cell membrane.

### Introduction

Pyocyanine, the blue pigment produced by many strains of *Pseudomonas aeruginosa*, has long been known to stimulate respiration. Friedheim (6, 7) observed that pyocyanine could increase the respiration of living cells and noted that its effect was not species specific, rabbit red blood cells and several species of bacteria being affected. Dickens (4), studying the metabolism of tissues, concluded that, in addition to acting as a respiratory catalyst, pyocyanine might also have an independent specific inhibitory effect on glycolysis. Schoental (12) found pyocyanine to be antibacterial, noting that the pigment inhibited the oxygen uptake of both *Vibrio cholerae* and *Staphylococcus aureus*. Subsequently many other workers, Waksman and Woodruff (14), Stokes *et al.* (13), and Young (15), studied the antibiotic activity of pyocyanine but found it to be unsuitable for therapeutic purposes. The inhibitory properties of pyocyanine were again noted by Judah and Williams-Ashman (9) in studies with rabbit-kidney cyclophorase when pyocyanine inhibited oxidative phosphorylation. Case and McIlwain (3) found that  $3.3 \times 10^{-3}$  M pyocyanine inhibited both oxygen and phosphate uptake and also depressed the P/O ratio of mammalian brain tissue.

The pigment has the characteristic reversible oxidation-reduction properties of the quinones and a fundamental structure approaching that of riboflavin. When reduced, pyocyanine also forms a free radical stable in aqueous solution and has the structure analogous to oxime, which permits it to form bidentate complexes with divalent ions. Moos (11) has shown that pyocyanine complexes with copper and concluded that it was the reduced form of the pigment which was involved.

In spite of the voluminous observations made on the biological activity of pyocyanine, no data are available on its mechanism of stimulation or inhibition. The present work was undertaken in an effort to determine the mechanism by which pyocyanine interferes with oxidations carried out by bacteria.

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### Materials and Methods

*Pseudomonas aeruginosa* (ATCC 9027) was used to produce the pyocyanine and *Pseudomonas fluorescens* (A 312) was used in the majority of the experimental work. Strains of *Escherichia coli* and *Proteus vulgaris* were used in confirmatory studies.

In order to produce pyocyanine, cultures of *P. aeruginosa* were grown in Roux flasks containing 100 ml. of the medium of Burton *et al.* (2). Cultures were incubated at 30° C. for four days, then centrifuged in a Sharples centrifuge. The supernatant was retained and the pyocyanine extracted from it, following the procedure of Elema and Sanders (5). This method yielded crystals of pyocyanine hydrochloride, which were dried in vacuum, bottled, and stored over silica gel at -10° C. For experimental work aqueous solutions of pyocyanine hydrochloride were prepared, neutralized, and stored at 6° C. Some precipitation occurred at 6° C., so the solutions were warmed to 30° C. for a period of 10 minutes before use.

Resting cells of *P. fluorescens* were prepared from 20-hour cultures, grown at 30° C. at pH 7.2 in Roux flasks. The medium consisted of ammonium dihydrogen phosphate, 0.3%; dipotassium hydrogen phosphate, 0.2%; yeast extract, 0.1%; iron as FeSO<sub>4</sub>, 0.5 p.p.m.; glucose, 0.3%; and magnesium sulphate heptahydrate, 0.1%. Washed cells obtained from 100 ml. of culture were resuspended in 6.0 ml. of M/30 phosphate buffer at pH 7.0.

Cultures of *E. coli* and *P. vulgaris* were grown in flasks containing 100 ml. of yeast tryptic digest broth, at 30° C., on a mechanical shaker. Resting cell suspensions were prepared as with *P. fluorescens*. Cell extracts of *E. coli* were prepared by subjecting a heavy suspension of washed cells to sonic oscillation in a 10 kc. Raytheon oscillator. The treated cells were then centrifuged at 18,000 g in the cold.

Warburg studies were carried out in the small (7 ml.) cups. A typical reaction vessel contained 0.5 ml. M/15 phosphate buffer pH, 6.8; 1.66  $\mu$ M. of substrate in 0.1 ml.; 375  $\mu$ g. of pyocyanine in 0.1 ml. of solution (this amount was designated "excess" pyocyanine); and water to a total volume of 1.0 ml. The center well contained 0.1 ml. of 20% KOH.

When *P. fluorescens* was the test organism, glucose-grown cells were used in experiments where glucose, gluconate, and 2-ketogluconate were to be the Warburg substrates. However, in the case of lactate, fumarate, and succinate, the growth substrate was the same as the Warburg substrate. Glutamate-grown cells were used when malate was to be the Warburg substrate. With *E. coli* and *P. vulgaris* glucose-grown cells were used.

Materials used to attempt reversal of pyocyanine inhibition were: MgSO<sub>4</sub> · 7H<sub>2</sub>O, MnCl<sub>2</sub> · 4H<sub>2</sub>O, FeSO<sub>4</sub> · 7H<sub>2</sub>O, cysteine hydrochloride, cystine, flavine mononucleotide (FMN), adenosine triphosphate (ATP), ethylenediaminetetraacetic acid (EDTA), and 8-hydroxyquinoline. Cells were incubated with these agents for 10 minutes prior to addition of pyocyanine. Amounts of these substances used were equimolar to the substrate.



The effect of pyocyanine on the phosphorylation of glucose by purified yeast hexokinase was determined by the method of Barker and Lipmann (1). Activity of glucose-6-phosphate dehydrogenase was followed by observing the reduction of TPN at 340 m $\mu$  in a Beckman DU spectrophotometer.

The identity of the compound accumulating as a result of the inhibition was determined by paper chromatography. A large Warburg cup was used containing 30 times the amounts of materials listed for the 7 ml. cups. Tris buffer at pH 7.4 was substituted for the phosphate buffer. This flask, containing excess pyocyanine, was incubated at 30° C. until the oxygen uptake in a control pair of conventional Warburg cups indicated that the reaction had run to completion. The contents of the large flask were then centrifuged at 6° C. for 15 minutes. The supernatant was removed and evaporated at 30° C. under vacuum to about 2.0 ml. total volume. This concentrated supernatant was analyzed by paper chromatography. The solvent system consisted of isopropanol, 70 ml.; ammonium hydroxide, 10 ml.; water, 20 ml. Two sprays were used for the detection of the compounds. The dried sheets were sprayed with silver nitrate (0.1 *N*) in 5 *N* ammonium hydroxide, placed in the dark at 30° C., and allowed to dry and develop overnight. The molybdate spray of Hanes and Isherwood (8) for the detection of phosphate compounds was also used.

### Results and Discussion

The measured oxygen uptake during the oxidation of glucose and 2-ketogluconate (1.66  $\mu$ M. of each) by resting cells of *P. fluorescens* was 148  $\mu$ l. and 112  $\mu$ l. respectively. When pyocyanine was present, glucose oxidation levelled off at 38  $\mu$ l. of oxygen uptake, while 2-ketogluconate oxidation was completely inhibited (Fig. 1). Such conditions therefore provided for the inhibition of glucose oxidation at the 1 micromole of oxygen level (38  $\mu$ l.) presumably at 2-ketogluconate.

In order to confirm that the inhibition by excess pyocyanine at 1  $\mu$ M. of oxygen uptake is actually inhibition at 2-ketogluconate, respiratory quotients were run. With an excess of pyocyanine, glucose oxidation required 1  $\mu$ M. of oxygen and no CO<sub>2</sub> was evolved. Paper chromatography was carried out to determine the identity of this intermediate. The molybdate spray did not reveal any spots indicative of a phosphorylated derivative; glucose-1-phosphate was used as a positive control. The silver nitrate spray revealed a very dense spot corresponding to 2-ketogluconate in color and *R<sub>f</sub>* value of 0.473.

In view of the fact that pyocyanine had been shown to have chelating properties, it was postulated that the inhibitory action of the dye was due to this characteristic. It was found that the common chelating compound, 8-hydroxyquinoline, also stopped the oxidation of glucose at 1  $\mu$ M. of oxygen. By the use of low concentrations of the two compounds it was further shown that the inhibitory actions of pyocyanine and 8-hydroxyquinoline were additive (Fig. 2). This would indicate that both were acting at a common

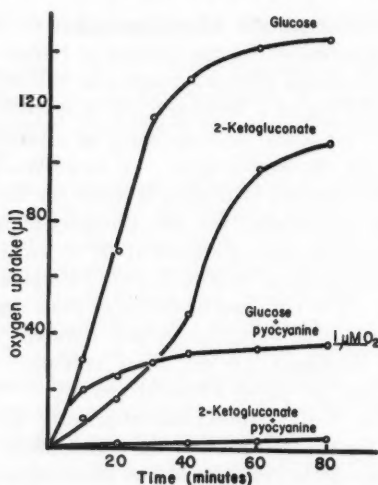


FIG. 1. Pyocyanine inhibition of glucose and 2-ketogluconate oxidation by *P. fluorescens*. Each Warburg vessel contained: 0.2 ml. of cell suspension; 0.5 ml. *M/15* phosphate buffer, pH 6.8; 1.66  $\mu$ M. substrate; 0.1 ml. 20% KOH; and water to 1.1 ml. Where indicated, 375  $\mu$ g. of pyocyanine was added.

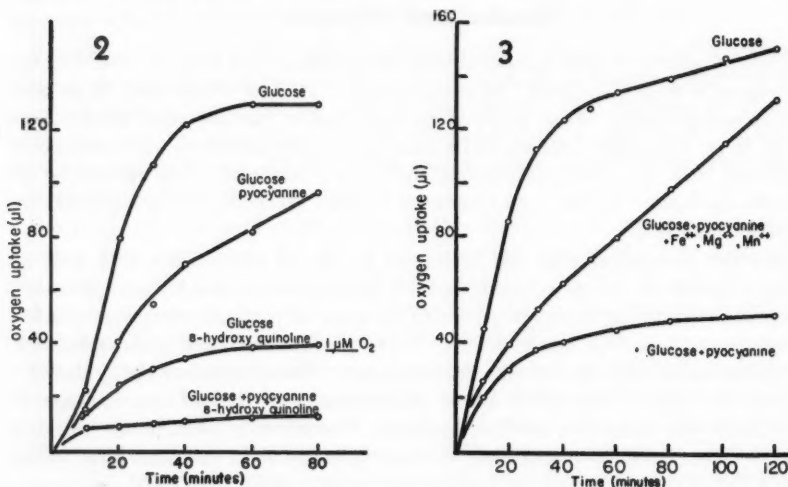


FIG. 2. Additive inhibitory effect of pyocyanine and 8-hydroxyquinoline on glucose oxidation by *P. fluorescens*. Warburg vessels contained: 0.2 ml. of cell suspension; 0.5 ml. *M/15* phosphate buffer of pH 6.8; 1.66  $\mu$ M. of glucose; and 0.1 ml. of 20% KOH. Pyocyanine (31.25  $\mu$ g.) or 8-hydroxyquinoline (200  $\mu$ g.) or both were added as indicated. The total volume was brought to 1.1 ml. with water.

FIG. 3. Reversal of pyocyanine inhibition of glucose oxidation by divalent cations. Each Warburg vessel contained: 0.2 ml. of cell suspension of *P. fluorescens*; 0.5 ml. *M/15* phosphate buffer of pH 6.8; 1.66  $\mu$ M. of glucose; and 0.1 ml. of 20% KOH. Inhibition was effected by adding 375  $\mu$ g. of pyocyanine. Reversal was shown when a mixture of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , and  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  containing 1.66  $\mu$ M. of each was added. Water was added to a total volume of 1.1 ml.

point and presumably binding an essential metal. Attempts were therefore made to bring about the reversal of this inhibition by the addition of metal ions.

Preliminary experiments indicated that the inhibition brought about by "excess" pyocyanine was not reversible. A level of dye that did not give as complete an inhibition was therefore chosen. This amount was 62.5  $\mu\text{g}$ . per ml. of reaction fluid and it resulted in an almost complete inhibition of glucose oxidation beyond the 1  $\mu\text{M}$ . of  $\text{O}_2$  per  $\mu\text{M}$ . of glucose level. With this concentration of pyocyanine the addition of equimolar concentrations of magnesium, manganese, and iron partially restored the rate of oxidation and allowed the reaction to proceed to completion (Fig. 3). Further investigation revealed that magnesium was the most effective cation although iron or manganese brought about some reversal.

The addition of cysteine, cystine, flavin mononucleotide, adenosine triphosphate, or ethylenediaminetetraacetic acid did not cause any reversal of inhibition.

It seems to be generally true that the oxidation of keto acids requires the presence of magnesium ions. It was decided therefore to determine the ability of pyocyanine to stop the oxidation of various substrates at a keto-acid intermediate. The substrates chosen were lactate, malate, succinate, and fumarate. The oxidation of both lactate and malate was inhibited after the uptake of 1 microatom of oxygen, indicating a block at pyruvate and oxalacetate respectively (Fig. 4). A respiratory quotient on the lactate inhibition showed no  $\text{CO}_2$  evolution. The oxidation of succinate and fumarate

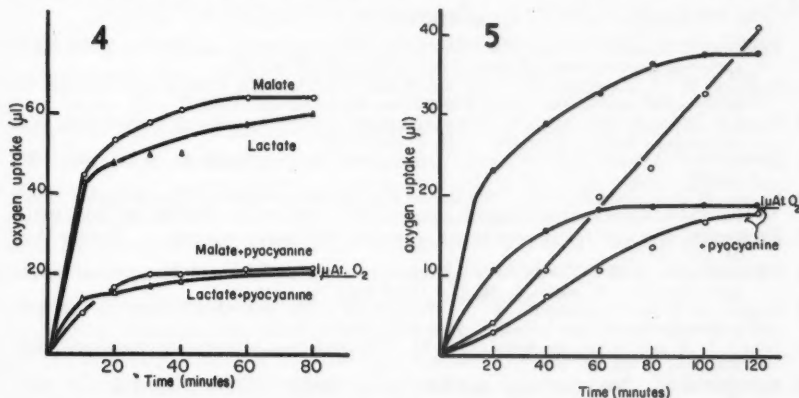


FIG. 4. Pyocyanine inhibition of malate and lactate oxidation by *P. fluorescens*. Warburg vessels contained: 0.2 ml. of cell suspension; 0.5 ml. *M*/15 phosphate buffer of pH 6.8; 1.66  $\mu\text{M}$ . substrate; 0.1 ml. 20% KOH; and water to 1.1 ml. Where indicated, 375  $\mu\text{g}$ . of pyocyanine was added.

FIG. 5. Pyocyanine inhibition of lactate oxidation by whole cells of *P. vulgaris* and by an extract of *E. coli*. Warburg vessels with *P. vulgaris* contained: 0.2 ml. of cell suspension; 0.5 ml. *M*/15 phosphate buffer of pH 6.47; 1.66  $\mu\text{M}$ . of lactate; 0.1 ml. 20% KOH; where required 750  $\mu\text{g}$ . of pyocyanine; and water to 1.1 ml. In the *E. coli* experiments, 0.2 ml. of sonic extract of *E. coli* was substituted for the cell suspension and the concentration of pyocyanine was reduced to 375  $\mu\text{g}$ . Solid circles represent *E. coli*; hollow circles, *P. vulgaris*.

was often stopped at oxalacetate; however, there were enough exceptions to indicate that the enzymes catalyzing these reactions sometimes prevented pyocyanine from effectively removing all the magnesium.

In an effort to show the general usefulness of pyocyanine as an inhibitor, its effect on other organisms was tested. The oxidation of lactate by whole cells of *P. vulgaris* levelled off at 18  $\mu$ l. of oxygen uptake in the presence of 750  $\mu$ g. of pyocyanine (Fig. 5); also the oxidation of lactate by extracts of *E. coli* was inhibited by 375  $\mu$ g. of pyocyanine at 18  $\mu$ l. of oxygen (Fig. 5). Hence in both cases oxidation was inhibited at pyruvate. Resting cells of *E. coli* were not affected by the dye and so it was necessary to make cell extracts. It appears therefore that the dye cannot penetrate the cell membrane of this organism. Resting cells of *Bacillus cereus* and a strain of *Achromobacter* also were not affected. Attempts to obtain active cell extracts of these organisms were not successful.

Other attempts to extend the uses of the inhibitor met with some success. It was found that glucose-6-phosphate dehydrogenase (Sigma) was inhibited by low concentrations of the dye, thus confirming the suggestion of Kornberg (10) that this enzyme is magnesium or manganese dependent. In contrast, yeast hexokinase, which is known to require magnesium, was not inhibited. This is in line with the recognized inability of fluoride to inhibit hexokinase and indicates that the enzyme has a very strong ability to bind magnesium ions.

It appears that pyocyanine may be a useful addition to our selection of inhibitors which act at a specific metabolic site.

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## ON THE NATURE OF THE VITAMIN B<sub>12</sub> REQUIREMENT IN SOIL BACTERIA ISOLATED BY LOCHHEAD AND HIS CO-WORKERS<sup>1</sup>

J. E. FORD<sup>2</sup> AND S. H. HUTNER<sup>3</sup>

### Abstract

Of 19 isolates of vitamin-B<sub>12</sub>-requiring soil bacteria, representing 10 distinct types, only one, "No. 12", responded to Factor A or B or pseudovitamin B<sub>12</sub>. Growth of several of the organisms was retarded by relatively high concentrations of pseudovitamin B<sub>12</sub>. Vitamin B<sub>12III</sub> was active for all but generally less so than cyanocobalamin.

The potential usefulness of these organisms as research tools is discussed, together with the ecological implications of the discovery by Lochhead and co-workers of the abundance of vitamin-B<sub>12</sub>-requiring bacteria in the soil.

### Introduction

Vitamin B<sub>12</sub> occurs in nature in several forms, closely analogous in their chemical structures, but widely different in their utilizability by different organisms. The compounds so far characterized are made up of a porphyrin-like nucleus built around an atom of cobalt—which occurs naturally as Factor B—and a nucleotide moiety. According to the nature of the nucleotide base, they seem to fall naturally into two groups: in one, containing vitamin B<sub>12</sub> and vitamin B<sub>12III</sub>, the bases are analogues of benziminazole; in the other group, represented by pseudovitamin B<sub>12</sub> and Factor A, the nucleotide contains a purine analogue.

The B<sub>12</sub> vitamins are synthesized by a wide range of microorganisms, and occur in natural materials subjected to bacterial action. At the same time, many microorganisms depend upon an exogenous supply of "vitamin B<sub>12</sub>". Some of these, like the higher animals, are narrowly specific in their requirement for the classical vitamin B<sub>12</sub>. Others are less exacting, and can make use of the purine-containing analogues, or of a physiological equivalent of the vitamin (e.g., methionine in *Escherichia coli* 113-3, and deoxyribosides in certain lactobacilli). A closely analogous situation is found among the vitamin-B<sub>12</sub> synthesizers: some can manufacture a variety of B<sub>12</sub>-vitamins; others seem to produce only cobalamin. Details of the vitamin B<sub>12</sub> requirements of different assay organisms are given elsewhere (1).

Much of the information available on the specificity of action of vitamin B<sub>12</sub> relates to the requirements of the four species commonly used for assay purposes—*Escherichia coli* 113-3, *Lactobacillus leichmannii*, *Ochromonas*

<sup>1</sup>Manuscript received October 5, 1956.

Contribution from the National Institute for Research in Dairying, Shinfield, Reading, England, and Haskins Laboratories, 305 E. 43rd St., New York 17, N.Y.

<sup>2</sup>National Institute for Research in Dairying.

<sup>3</sup>Haskins Laboratories.

Had circumstances permitted, Dr. Lochhead would have been invited to share in the authorship of a paper along the lines of this one. We hope this paper and the accompanying one on vitamin-B<sub>12</sub>-requiring bacteria will make clear why our indebtedness to him cannot adequately be expressed in a footnote nor, for that matter, in the form of joint authorship of any one paper.

TABLE I  
SOME PROPERTIES OF NATURALLY OCCURRING B<sub>12</sub>-VITAMINS

Name of compound	Base of nucleotide	<i>E. coli</i> (plate test)	<i>E. coli</i> (tube test)	Microbiological activity*		
				<i>L. leichmannii</i> (tube test)	<i>E. gracilis</i> (tube test)	<i>O. malhamensis</i> (tube test)
Factor B	No nucleotide present	+++	+	—	—	—
Cyanocobalamin	5,6-Dimethylbenzimidazole	+++	+++	+++	+++	+++
Factor I,						
vitamin B <sub>12III</sub>	5-Hydroxybenzimidazole	+++	++	++	—	++
Pseudovitamin B <sub>12</sub>	Adenine	+++	+	++	++	—
Factor A	2-Methyladenine	+++	+	++	++	—
Factors C <sub>1</sub> and C <sub>2</sub>	Not known	+++	+	+	+	—
Factor D	Not known	—	—	—	—	—
Factor E	Not known	+++	—	—	—	—
Factor F	Not known	+++	+	—	—	—
Factor G	Hypoxanthine	+++	—	+++	—	—
Factor H	2-Methylhypoxanthine	+++	++	++	—	—

\*+++ denotes activity equivalent to that of cyanocobalamin.

++ denotes activity of the order of 50% that of cyanocobalamin.

++ denotes activity of the order of 10% that of cyanocobalamin.

— denotes activity less than 1% that of cyanocobalamin.

*malhamensis*, and *Euglena gracilis* var. *bacillaris*; it is collated broadly in Table I. There is need for a more detailed appraisal of other known vitamin-B<sub>12</sub>-requiring microorganisms.

Lochhead and Burton (9) isolated and characterized 30 soil bacteria, representing 10 morphological and physiological types, for which vitamin B<sub>12</sub> is an essential nutrilit. We have made a more detailed study of the requirement in 19 of these isolates, representing all of the 10 types. In this communication we describe growth tests with five of the B<sub>12</sub>-vitamins: Factor A, Factor B, pseudovitamin B<sub>12</sub>, vitamin B<sub>12III</sub>, and cyanocobalamin.

### Experimental

The test organisms were designated by the following culture numbers (type numbers in parenthesis):

7(1), 8(1), 10(1), 12(8), 17(2), 23(2), 27(1), 28(2), 30(7), 38(3),  
40(4), 54(9), 56(1), 61(1), 62(5), 76(6), 86(10), 100(3), 110(1).

### Maintenance of Cultures

Stock cultures were transferred monthly on a yeast-soil extract medium (9). Its composition, given here for convenience, was as follows: soil extract, 250 ml. (prepared by autoclaving 1 kg. field soil with 1 l. tap water for 30 minutes at 118–121° C., filtering after the addition of 2 g. CaCO<sub>3</sub>, and making the filtrate up to 1 l.); glucose, 0.75 g.; K<sub>2</sub>HPO<sub>4</sub>, 0.75 g.; KNO<sub>3</sub>, 0.375 g.; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.15 g.; CaCl<sub>2</sub>, 0.075 g.; NaCl, 0.075 g.; FeCl<sub>3</sub>·6H<sub>2</sub>O, 0.0075 g.; "Difco" yeast extract, 0.75 g.; agar, 10 g.; distilled water to 1000 ml.; pH to 6.8 with N HCl.

After transfer, the cultures were incubated 3 days at 30°, and then stored at 4°.

The medium used for tests on vitamin B<sub>12</sub> and the related compounds was made up at 5X final strength, to the following formula: glucose, 1 g.; K<sub>2</sub>HPO<sub>4</sub>, 1 g.; KNO<sub>3</sub>, 0.5 g., MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2 g.; CaCl<sub>2</sub>, 0.1 g.; NaCl,



0.1 g.; FeCl<sub>3</sub>·6H<sub>2</sub>O, 0.01 g.; "Difco" yeast extract, 1 g.; NaCN, 2 mg.; distilled water to 200 ml.; pH to 6.8. It was stored at -20° in polyethylene containers.

Cultures for use as inocula were grown in the assay medium, diluted to single strength, and then supplemented with 0.4 mμg. cyanocobalamin/ml. This enriched medium was dispensed in 5-ml. amounts into 19 × 150 mm. Pyrex test tubes, which were then plugged and sterilized by autoclaving for 15 minutes at 10 lb. pressure. After inoculation from the stock cultures, the tubes were shaken continuously in a 30° incubator for 24 hours or, with slower growing cultures, for 48 hours.

For pad-plate assays the basal medium, at single strength, was solidified with 1.5% agar. To 100 ml. portions of sterile, molten medium cooled to 45°, 1 ml. of inoculum was added. The medium then was dispensed without delay in 12-ml. portions on to sterile 10-cm. Petri dishes. Solutions containing 0.5 μg./ml. of one or other of the B<sub>12</sub>-vitamins were applied in 0.01 ml. amounts to disks of filter paper (Whatman No. 4) 8 mm. in diameter. These were dried in an air oven at 55° before being placed on the seeded agar in the usual manner. The dishes then were incubated for 48-72 hours at 30°.

Tube tests were set up in 19 × 150 mm. optically matched Pyrex test tubes. A standard solution of cyanocobalamin containing 0.2 mμg./ml. was added to paired tubes at levels of 0.5, 1, 2, and 4 ml. Solutions of vitamin-B<sub>12</sub>-like compounds, covering a range of concentrations from 0.2 mμg./ml. to 0.5 μg./ml. were added at the same levels, and where necessary water was added to the tubes to bring their fluid content to 4 ml. The tubes were "dosed" with 1 ml. of the 5× basal medium, plugged with cotton, and autoclaved for 10 minutes at 10 lb. pressure.

To each of the tubes then was added one drop of inoculum culture diluted fivefold with sterile, vitamin-B<sub>12</sub>-free medium. The tubes were incubated at 30° with continuous shaking for 36 or 72 hours, depending upon the rate of growth of the culture. After incubation, growth in the tubes was measured turbidimetrically in a Lumetron colorimeter (Photovolt Corporation, New York).

#### *Vitamin-B<sub>12</sub>-like Compounds*

We are indebted to Dr. K. Bernhauer (Biochemisches Laboratorium der Aschaffenburg Zellstoffwerke, A. G., Stockstadt-am-Main) for a sample of vitamin B<sub>12</sub>III, and to Dr. E. S. Holdsworth (N.I.R.D.) for samples of pseudovitamin B<sub>12</sub>, Factor A, and Factor B.

### Results

#### *Response to B<sub>12</sub>-Vitamins*

##### *(a) Pad-plate Tests*

Fig. 1 shows the response of cultures 27, 28, 100, 40, 62, 76, 12, 54, and 86, representing 9 of the 10 types, to the five B<sub>12</sub>-vitamins tested. Culture No. 30, representing type 7, grew very weakly in agar and we were not able to prepare a satisfactory plate.

With the exception of No. 30, all the isolates responded to cyanocobalamin and vitamin B<sub>12III</sub>, which gave zones of about the same diameter. For several of the isolates vitamin B<sub>12III</sub> was clearly less active, judging by the slower development of the growth zones and their lesser opacity. For Nos. 7, 8, 10, 12, 54, 76, 86, and 100 both forms of the vitamin appeared about equally active.

Only one of the isolates, No. 12, grew well with Factors A and B and pseudovitamin B<sub>12</sub>. For the remainder, the three compounds had little or no activity, although pseudovitamin B<sub>12</sub> evoked a faint response in cultures 7, 27, 54, 62, 76, 86, and 110.

These findings are summarized in Table II.

TABLE II  
ACTIVITIES OF VITAMIN B<sub>12</sub> AND RELATED COMPOUNDS FOR THE  
SOIL ISOLATES, ASSESSED IN PAD-PLATE TESTS

Isolate number	Vitamin B <sub>12</sub>	Vitamin B <sub>12III</sub>	Factor A	Factor B	Pseudo-vitamin B <sub>12</sub>
7	+++	+++	—	—	v.f.*
8	+++	+++	—	—	—
10	+++	+++	—	—	—
12	+++	+++	+++	+++	+++
17	+++	++	—	—	—
23	+++	++	—	—	—
27	+++	++	—	—	v.f.*
28	+++	++	—	—	—
38†	+++	++	—	—	—
40	+++	++	—	—	—
54	+++	+++	—	—	v.f.*
56	+++	++	—	—	—
61	+++	++	—	—	—
62	+++	++	—	—	v.f.*
76	+++	+++	—	—	v.f.*
86	+++	+++	—	—	v.f.*
100	+++	+++	—	—	—
110	+++	++	—	—	v.f.*

\*"v.f." denotes very faint, diffuse zones of exhibition.

†Studied in detail in Ref. 5.

#### (b) Tube Tests

For the tube tests the organisms were divided arbitrarily into two groups. For one, comprising the faster-growing cultures, the assay tubes were incubated for 36 hours. The slower-growing organisms in the other group were incubated for 72 hours.

Slow-growing cultures: 17, 23, 28, 30, 40, 54, 61, 62, 76, 100.

Fast-growing cultures: Nos. 7, 8, 10, 12, 27, 56, 86, 110.

Table III shows the activities of pseudovitamin B<sub>12</sub>, Factor B, and vitamin B<sub>12III</sub> for the different test organisms. We had not enough pure Factor A for use in these tube tests. The findings were not precisely reproducible,

PLATE I

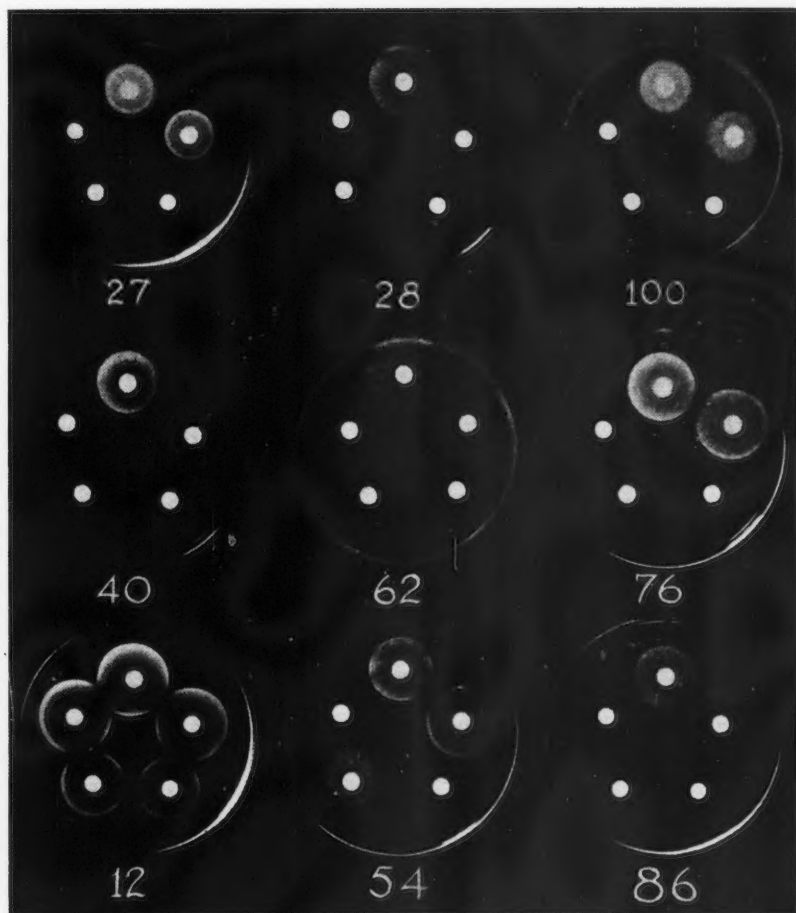


FIG. 1. Response of cultures of B<sub>12</sub>-vitamins. Reading clockwise from the top of each plate: vitamin B<sub>12</sub>, vitamin B<sub>12</sub>III, Factor A, pseudovitamin B<sub>12</sub>, and Factor B.

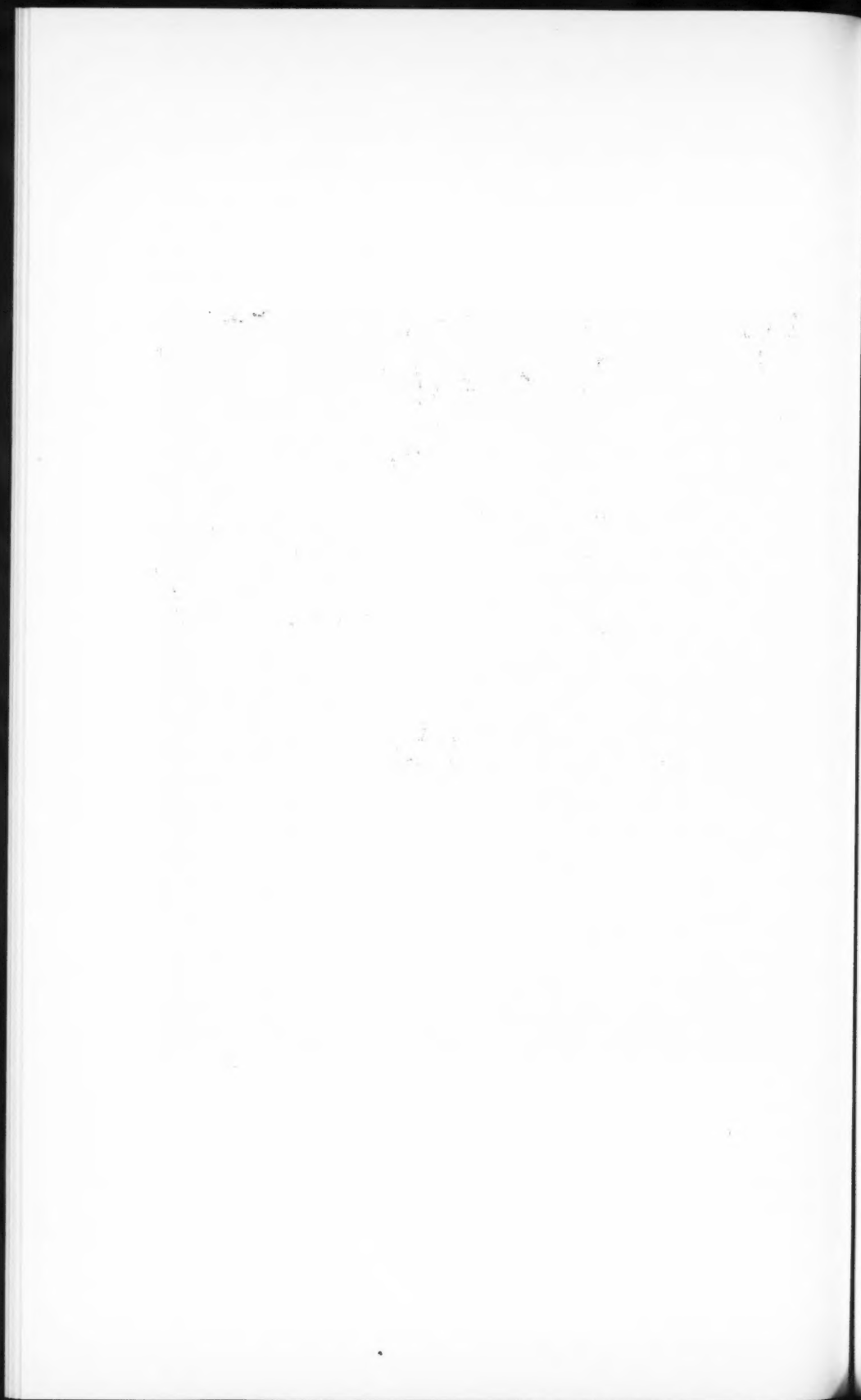


TABLE III  
RELATIVE ACTIVITIES\* OF VITAMIN B<sub>12</sub> AND RELATED COMPOUNDS  
MEASURED IN TUBE TESTS

Isolate number	Vitamin B <sub>12</sub> III	Pseudovitamin B <sub>12</sub>	Factor B
7	22	0	0
8	92	0	0
10	40	0	0
12	120	80	90
17	42	0	0
23	41	0	0
27	25	0	0
28	16	0	0
30	75	0	0
38	31	0	0
40	40	0	0
54	70	0	0
56	13	0	0
61	30	0	0
62	160	0	0
76	50	0	0
86	130	0	0
100	9	0	0
110	15	0	0

\*Numerical values have been assigned with some misgivings, as even under carefully standardized conditions of test the results were not precisely reproducible. The activity of vitamin B<sub>12</sub> is taken as 100.

even under carefully standardized conditions of test, and the figures should be regarded as approximations. Thus, in the series of tests on vitamin B<sub>12</sub>III the following results were obtained:

Culture No.	Activity of vitamin B <sub>12</sub> III*
8	100, 143, 85, 91
10	60, 52, 44, 36
56	15, 10, 10, 16
86	150, 150, 110, 110
110	21, 14, 13, 17

\*Vitamin B<sub>12</sub> = 100.

#### Tests for Synergism and Antagonism

All the isolates except No. 12 resembled the protozoan *Ochromonas malhamensis* in their patterns of response to the B<sub>12</sub> vitamins. It has been shown recently (3) that pseudovitamin B<sub>12</sub> and Factor A, though intrinsically inactive for *Ochromonas*, may depress the uptake of vitamin B<sub>12</sub>, and thereby inhibit growth; Factor B, on the other hand, is not taken up by *Ochromonas*, and does not antagonize vitamin B<sub>12</sub>. It appears that the accumulation of vitamin B<sub>12</sub> from the surrounding medium involves the combination of the vitamin with a specific cell-protein. Saturation of this protein with Factor A or pseudovitamin B<sub>12</sub> precludes the uptake of vitamin B<sub>12</sub>. The simple picture is complicated in that, at relatively low concentrations, pseudovitamin

B<sub>12</sub> and Factor A actually enhance the activity of vitamin B<sub>12</sub>. This contradictory behavior apparently is connected with the presence of traces of the vitamin-B<sub>12</sub>-binding protein in the culture liquors. A fuller discussion of the mechanism for vitamin B<sub>12</sub> uptake in *Ochromonas* will be attempted elsewhere. At this stage, we were interested to test the effects of pseudovitamin B<sub>12</sub> and Factor B on the responses of the soil isolates to growth-limiting concentrations of vitamin B<sub>12</sub>.

To two series of paired tubes were added 4-ml. amounts of solutions of pseudovitamin B<sub>12</sub>, containing per ml. 0.0004, 0.002, 0.01, 0.05, 0.25, or 1.25  $\mu$ g. of the analogue. Two "blanks" were dosed with 4 ml. of water.

Another two series of tubes were set up with solutions of Factor B.

To each of the tubes was now added 1 ml. of 5 $\times$ -strength medium, enriched with 0.001  $\mu$ g. vitamin B<sub>12</sub>/ml. After being plugged and sterilized, the set of tubes were inoculated and incubated. This whole procedure was carried out for each of the isolates excepting No. 12. For the faster-growing isolates, one of each of the two series was read after 40 hours' incubation and the other after 64 hours. For the slower-growing isolates the corresponding periods were 48 and 73 hours.

Fig. 2 illustrates the procedure more clearly, showing the effect of pseudovitamin B<sub>12</sub> on the growth of culture No. 10. At the lower concentrations the analogue proved slightly stimulatory, but at the higher concentrations it retarded growth.

A broadly similar picture was obtained with isolates 8, 10, 54, and 56, although with No. 56, the synergism of vitamin B<sub>12</sub> and pseudovitamin B<sub>12</sub> was much more marked. With Nos. 7, 17, 23, 27, 28, 30, 38, 61, 62, and 110 no stimulation of growth was observed at the lower concentrations of

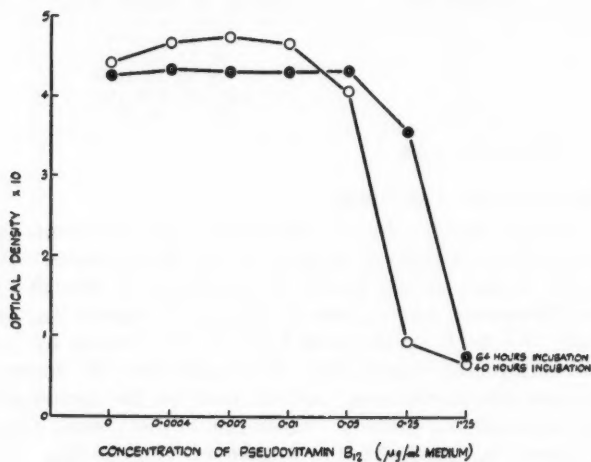


FIG. 2. Effect of pseudovitamin B<sub>12</sub> on the growth of No. 10.



pseudovitamin B<sub>12</sub> but the higher concentrations were markedly inhibitory. For No. 86, the analogue was stimulatory up to the highest concentration tested. With Nos. 40 and 76 no significant effect was found.

The isolates should not be compared too closely on the basis of the above findings. The effect of pseudovitamin B<sub>12</sub> was transient, except possibly at the highest concentration, and depended critically on the test conditions: concentration of vitamin B<sub>12</sub>, period of incubation, stage of growth, and amount of inoculum; and doubtless on other factors. Indeed, it seems likely that most of the isolates behave in essentially the same fashion, and differ only quantitatively in their susceptibility to inhibition by pseudovitamin B<sub>12</sub>.

For most of the isolates Factor B was quite inert within the wide range of concentrations tested, but in a few instances a slight synergism was apparent at the highest test level.

### Discussion

#### *Analytical Considerations*

Several of these soil isolates have promising characteristics as reagents for vitamin B<sub>12</sub>. They are fast growing and animal-like in their patterns of response to the B<sub>12</sub>-vitamins; and unlike *Ochromonas* they grow well in agar, and so can be used in the convenient pad-plate technique. It can be shown, however, that the tube assay techniques discriminate much more effectively against the 'inactive' analogues, whose presence in relatively high concentration with vitamin B<sub>12</sub> gives rise to larger and correspondingly less dense zones of exhibition in the plate assays.

Consideration of the physical mechanism of the plate test, and of the possibility that the 'inactive' analogues may slow down the rate at which vitamin B<sub>12</sub> is absorbed and utilized (cf. 3), suggests an explanation for this effect. The vitamin is presented only transiently to the bacteria in the agar. If both active and 'inactive' forms combine to satisfy the immediate absorptive capacities of the bacteria, then some of the active form will be free to diffuse further than would be possible if the 'inactive' analogues were not present.

For several of the isolates the activity of vitamin B<sub>12III</sub> varies according to the conditions of test. In plate tests the compound may be about as potent as vitamin B<sub>12</sub> when judged only by the diameters of the zones of growth elicited. On the other hand the zones of exhibition may develop more slowly during incubation, and be generally of lesser opacity. In such instances it is found that the tube test gives a much lower estimate of relative potency. Neither measure is strictly meaningful, but for the practical purpose of assessing the vitamin B<sub>12</sub> activity of food stuffs, there might be advantage in the method least responsive to vitamin B<sub>12III</sub>.

Isolate No. 12 responds about equally to Factors A and B, pseudovitamin B<sub>12</sub>, and vitamins B<sub>12</sub> and B<sub>12III</sub>, both in cup-plate and in tube assays. Furthermore, the organism seems highly specific for vitamin-B<sub>12</sub>-group compounds and, unlike *E. coli* 113-3, is unaffected by methionine. This

suggests useful applications to the assay of "Total B<sub>12</sub>". Vitamin B<sub>12</sub> analogues and Factor B, while not active for higher animals, may be important in the ecology of such environs as the rumen, soil, freshwater bodies, and, as has now emerged as probable, the sea (7)—all of which points to potential applications for No. 12 or its like. A direct ecological role for Factor B in the sea is suggested by the observation that a vitamin B<sub>12</sub> requirement of the common planktonic diatom *Skeletonema costatum* is satisfied by Factor B (2).

Factor B is fairly stable to acid hydrolysis—a procedure used in its preparation by removal of the nucleotide moiety from the cobalamin molecule and from the vitamin analogues (13). Such drastic procedures have found no place in the preparation of extracts of natural materials for assay. But for the assay of "total B<sub>12</sub>", and for studies of 'bound' forms, a technique based on the conversion of the B<sub>12</sub> vitamins to this 'common denominator'—Factor B—might be advantageous.

As the vitamin B<sub>12</sub> requirement in all the soil isolates is demonstrable even in the presence of rather high concentrations of yeast extracts or of plant digests (e.g. Phytone, B.B.L.), one might suppose that basal media for assay purposes could be prepared simply from such materials instead of by preparing complex chemically defined media. Preliminary findings in the American author's laboratory indicate that these crude natural materials may have limiting amounts of available substrates and of inorganic nutrients such as calcium. As yet, neither wholly 'synthetic' media nor media based on crude natural materials are as dependable as could be desired.

#### *Uptake of B<sub>12</sub>-Vitamins*

The antagonism observed in several instances between pseudovitamin B<sub>12</sub> and cyanocobalamin parallels similar observations with *Ochromonas* (3). A broad question is whether the different activities of the B<sub>12</sub>-vitamins for different organisms reflect in some manner the tenacity of the binding protein-vitamin bond (1). A corollary question is whether vitamin-B<sub>12</sub>-“trapping” mechanisms always involve the specific mucoproteins (8). To help elucidate the subtle differences which are responsible for selective utilization, one would welcome an extreme case: an organism responding to Factor A or pseudovitamin B<sub>12</sub> and not to cobalamin. Why no such organism has yet been isolated gives cause for wonder. It has however been reported that *Lactobacillus acidophilus* ATCC 832 responds more sensitively to pseudovitamin B<sub>12</sub> than to cyanocobalamin (11). The “non-selective” isolation procedure developed by Lochhead *et al.*, which revealed the abundance of B<sub>12</sub>-auxotrophs in the soil, embodied the implicit assumption, reasonable in the light of knowledge at that time, that the soil extract contained in the primary isolation medium carried a representative assortment of B<sub>12</sub>-vitamins. If the extract had contained a preponderance of cobalamin, then organisms responding preferentially to cobalamin would have been favored.

Perhaps a more promising source of microorganisms responding preferentially to analogues of vitamin B<sub>12</sub> is the rumen of cattle or sheep. About

70% of the "vitamin B<sub>12</sub>" in the calf rumen is in the form of Factor A, and in the sheep rumen the vitamin B<sub>12</sub> activity measured with *E. coli* was up to 20 times that measured with *Ochromonas* (6).

#### Vitamin B<sub>12III</sub>

The fact that this compound is active for all the isolates (Tables II and III) as well as for *Ochromonas* and higher animals is of interest. There is no evidence that it represents an essential stage in vitamin B<sub>12</sub> metabolism: in *Ochromonas*, *E. coli* 113-3, and in the chick it is utilized unchanged, without prior conversion to cobalamin. It has recently been shown that its nucleotide contains 5-hydroxybenzimidazole (4, 12), in contrast with the dimethylbenzimidazole present in cobalamin.

### Concluding Remarks

We have sought to sketch some of the ways in which the studies initiated by Dr. Lochhead may open new windows to the understanding of the ecology of natural waters, soil, and animals. He has shown that soil is a fertile source of microbial tools for the investigation of hitherto inaccessible cellular processes. We need only remind ourselves of his demonstration (10) that 1 g. of soil contains more than 3,700,000 bacteria requiring vitamin B<sub>12</sub> in order not to worry about a scarcity of B<sub>12</sub>-reagents—not to mention the puzzles they exemplify!

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## NUTRITION OF A COBALAMIN-REQUIRING SOIL BACTERIUM<sup>1</sup>

MILTON K. GOLDBERG,<sup>2</sup> S. H. HUTNER,<sup>3</sup> AND J. E. FORD<sup>3</sup>

### Abstract

The pattern of the vitamin B<sub>12</sub> requirement of a soil bacterium—"Lochhead 38" (provisionally assigned to *Arthrobacter*) resembled that of the protozoan *Ochromonas malhamensis* and of higher animals. Of the naturally-occurring B<sub>12</sub>-vitamins, cyanocobalamin and vitamin B<sub>12III</sub> are active. Pseudovitamin B<sub>12</sub> and Factor A have very little or no intrinsic activity, and when present in relatively high concentrations both compounds depress the rate of the growth response to limiting cyanocobalamin. Factor B, the porphyrin-like nucleus of the vitamin B<sub>12</sub> molecule without the nucleotide, is inactive, as are also methionine and deoxyribosides. A disadvantage in the use of Lochhead 38 for assay purposes is that in vitamin-B<sub>12</sub>-deficient cultures the organisms flocculate.

### Introduction

Lochhead and Thexton (9) drew attention to the occurrence in soil of an important group of bacteria that require vitamin B<sub>12</sub>. The need for the vitamin persisted even in the presence of 0.1% yeast extract. This implied that methionine or deoxyribosides cannot replace vitamin B<sub>12</sub>.

We have studied in some detail the nutrition of one of these bacteria, especially the nature of the vitamin B<sub>12</sub> requirement. The requirement for the vitamin B<sub>12</sub> molecule itself proved to be much like that of *Ochromonas malhamensis* and higher animals, but certain disadvantages attend its use as an assay organism. The present paper is therefore more a description of some of its nutritional features than a detailed recommendation and guide for its practical use.

### Materials and Methods

We are indebted to Dr. Lochhead for the organism. It is assigned provisionally to the genus *Arthrobacter*, and is designated as Type 3, No. 38 (8). Our thanks are due to Dr. K. Bernhauer (Biochemisches Laboratorium der Aschaffenburg Zellstoffwerke, A. G., Stockstadt-am-Main) for a sample of vitamin B<sub>12III</sub> and to Dr. E. S. Holdsworth (N.I.R.D.) for samples of pseudovitamin B<sub>12</sub>, Factor A, and Factor B; Dr. J. J. Piffner of Parke, Davis & Co. also supplied a sample of pseudovitamin B<sub>12</sub>.

Stock cultures were maintained on slopes of the basal medium (Table I), diluted to single strength and supplemented with vitamin B<sub>12</sub> 0.002 µg./ml., Trypticase (Baltimore Biological Laboratory) 0.1%, and agar 1.5%. The

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Contribution from the Haskins Laboratories, 305 E. 43 St., New York 17, N.Y., U.S.A.

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TABLE I

LIQUID BASAL MEDIUM FOR ASSAY OF VITAMIN B<sub>12</sub> WITH LOCHHEAD No. 38

Na <sub>2</sub> citrate.2H <sub>2</sub> O	0.05 g.	Biotin	0.4 µg.
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.025 g.	Thiamine HCl	0.1 mg.
KH <sub>2</sub> PO <sub>4</sub>	0.01 g.	Metals*	1.0 mg.
CaCO <sub>3</sub>	2.5 mg.	NaCN	0.2 mg.
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.01 g.	Distilled water to 100 ml.	
Sucrose	1.0 g.	pH 6.8-7.0 with Tris† buffer	
DL-Asparagine	0.2 g.		

NOTE: It was convenient to store the basal medium as a 5-fold concentrated solution. It should not be brought to final pH until all the ingredients are dissolved.

\*"Metals" were supplied as a dry mixture which contains in 1.0 mg.: Fe, 0.09 mg.; Zn, 0.045 mg.; Mn, 0.02 mg.; Cu, 0.0034 mg.; Co, 0.0045 mg.; B, 0.0045 mg.; V, 0.0022 mg.; Mo, 0.0022 mg. The Co, B, V, and Mo were added for the sake of completeness and are safe to omit.

†"Tris" buffer is tris(hydroxymethyl)aminomethane (Sigma Chemical Co.).

cultures were transferred monthly and after 3 days' incubation at 28-30° they were stored at 4°. Refrigerated cultures remained viable at least 6 months.

The organism is strongly aerobic and was grown in shallow culture for comparison of the various experimental media: 5-ml. portions were dispensed in 35 ml. "micro-Fernbach" borosilicate flasks (Kimble Glass Co.). The culture methods have been detailed elsewhere (1).

For tests of vitamin B<sub>12</sub> and the related compounds, the inocula were grown in the basal medium at single strength, supplemented with 0.4 mµg. vitamin B<sub>12</sub>/ml. The medium was dispensed in 5-ml. amounts into 19×150 mm. Pyrex test tubes, which were then plugged and sterilized by autoclaving for 15 minutes at 10-lb. pressure. After inoculating from a stock culture the tubes were shaken for 48 hours at 30°. One drop of this culture, undiluted, was used to inoculate each assay tube. The procedure followed in the tube tests was otherwise as recommended for the assay B<sub>12</sub> with *Ochromonas malhamensis* (3).

For pad-plate assays the basal medium was solidified with 1.5% agar. To 100-ml. portions of molten medium there was added, after cooling to 45°, 1 ml. of inoculum. The inoculated agar was then dispensed without delay in 12-ml. portions onto sterile 10-cm. Petri dishes. Suitable dilutions of the test solutions were applied in 0.01-ml. amounts to disks of filter paper (Whatman No. 4) 8 mm. in diameter. These were dried in air at 55° before they were placed on the seeded agar in the usual way. The plates were sealed with tape to prevent drying during the 3-day incubation period.

## Results

### Basal Medium

The basal medium (Table I) is complete in the sense that when supplemented with vitamin B<sub>12</sub> (1 mµg./ml. single-strength medium) the addition of complex natural materials—peptones, yeast, soil extract, or a crude extract



PLATE I

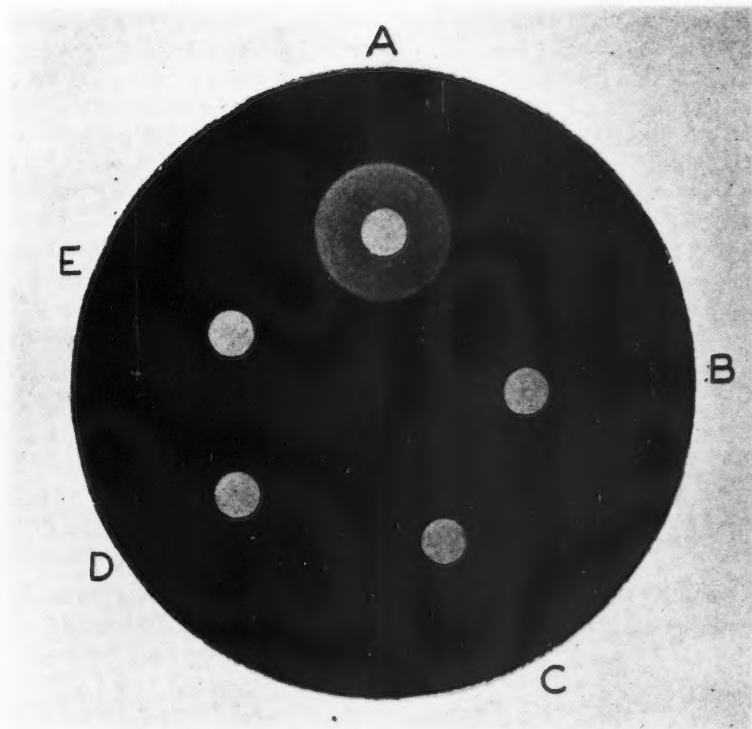
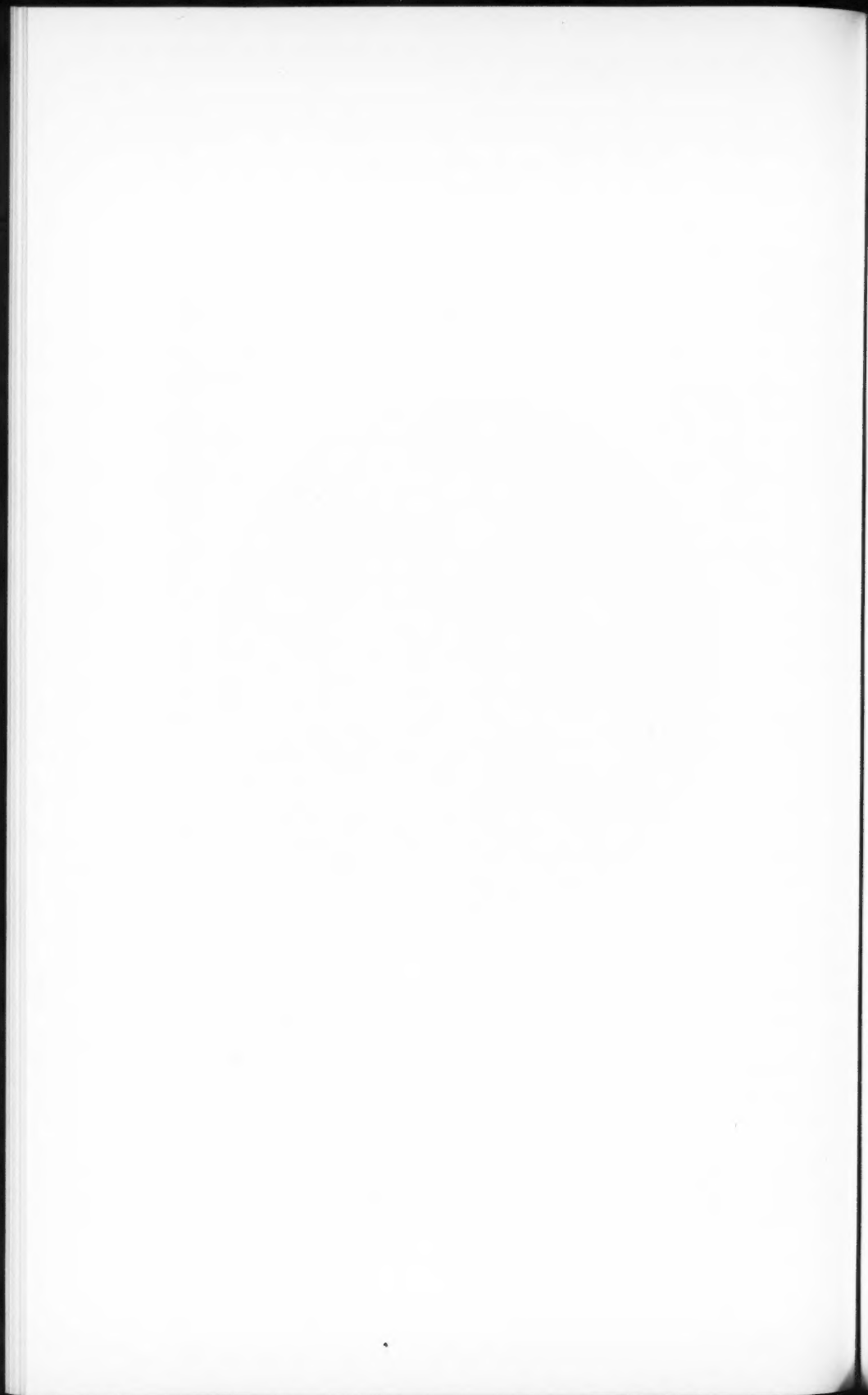


FIG. 1. Zones of response to  $B_{12}$ -vitamins on agar plates. A, vitamin  $B_{12}$ ; B, vitamin  $B_{12III}$ ; C, Factor A; D, Factor B; E, pseudovitamin  $B_{12}$ .



of liver—increased neither the growth rate nor the peak cell population. The optimum temperature for growth was 30°. At 32° the cultures grew only slowly, and at 35° there was no growth. The pH of the medium increased during growth of the cultures, reaching 8.0 to 8.4. Growth could not be initiated at pH 6.0.

### Substrates

Sucrose 1.0% + DL-asparagine 0.2% was the combination chosen after many experiments. The following compounds, tested in the range 0.05–0.2%, did not enhance growth when added to the liquid sucrose–asparagine medium: Na<sub>2</sub> succinate .6H<sub>2</sub>O, Na acetate .3H<sub>2</sub>O, Na H glutamate, DL-alanine, glycine, and (weights calculated as free acid) DL-aspartate, DL-lactate, and DL-malate, and acid hydrolyzates of gelatin and casein. On the complete medium solidified with 1.5% agar, and with nitrogen supplied as (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.05% while asparagine and carbohydrate were omitted from the basal medium, L-rhamnose and cellobiose supported about as good growth as did sucrose. Poorly-soluble substrates were sprinkled on the agar surface; soluble low-molecular substrates were applied as impregnated paper disks. The following were inert under these conditions:

Esculin	D-Galactose	Pectin (citrus)
Alginate acid	D-Galacturonic acid	Phloridzin
L-Arabinose	Gum tragacanth	Raffinose
Carrageenin	Glycogen	Salicin
Chitin	Inositol	Starch
K chondroitin sulphate	Inulin	Trehalose
L-Fucose	Lignin	Xylan

Acidic substrates (e.g., alginate acid) were ground with NaHCO<sub>3</sub> and the mixture autoclaved before application, so that the resulting preparations were neutral or slightly alkaline.

### Response to B<sub>12</sub>-Vitamins

In both tube and plate tests the growth responses can be related linearly over a wide range to the concentration of vitamin B<sub>12</sub>.

### Plate Tests

Fig. 1 shows the appearance of an agar plate to which several of the B<sub>12</sub>-vitamins had been applied in equivalent amounts. Vitamin B<sub>12</sub> itself gave rise to dense, sharply-defined zones of exhibition (Fig. 2). The response to vitamin B<sub>12</sub>III was slower to appear and the growth zones were often less dense and clearly defined. Factors A and B and pseudovitamin B<sub>12</sub> gave a very faint, diffuse halo of growth after prolonged incubation, but it is possible, and perhaps likely in view of the peculiar difficulty of isolating these compounds in pure form (7), that this may be attributable to traces of vitamin B<sub>12</sub> still present as an impurity.

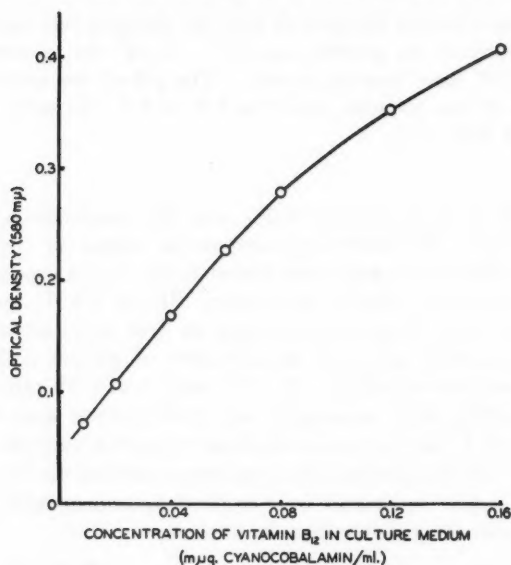


FIG. 2. Response of Lochhead 38 to graded concentrations of vitamin B<sub>12</sub>.

#### Tube Tests

Fig. 2 shows the response to graded amounts of vitamin B<sub>12</sub>. In tube assays, vitamin B<sub>12III</sub> was about 25% as active as vitamin B<sub>12</sub> under the test conditions employed. This low figure may in some measure reflect a slower utilization, but as prolonging the time of incubation did not greatly increase the measured potency, it seems likely that the compound is essentially less active than vitamin B<sub>12</sub> itself.

Factors A and B and pseudovitamin B<sub>12</sub> were inactive up to 0.5 μg./ml. culture medium. Factor B, even in relatively high concentration (0.2 μg./ml.), did not affect the response to limiting vitamin B<sub>12</sub>. Factor A and pseudovitamin B<sub>12</sub>, on the other hand, retarded the rate of growth at this high concentration. This pattern of response is similar to that shown by the protozoan *Ochromonas malhamensis*, in which vitamin-antivitamin relationships between vitamin B<sub>12</sub> and pseudovitamin B<sub>12</sub> or Factor A have been demonstrated (4).

With Lochhead 38, antagonisms between Factor A or pseudovitamin B<sub>12</sub> and vitamin B<sub>12</sub> can be inferred from the appearance of agar plates to which vitamin B<sub>12</sub> and the analogues have been applied in close proximity. Under these conditions the shape of the B<sub>12</sub>-zone, normally circular, is distorted.

The organism is not well suited for the assay of vitamin B<sub>12</sub> because frequently in the course of incubation the cultures flocculate and form a granular, non-dispersible type of growth. Chaplin and Lochhead (2) related this to an abnormal cell morphology associated with vitamin B<sub>12</sub> deficiency. So

long as a young inoculum is employed, flocculation does not complicate the use of the organism in agar culture, as in pad-plate assays or in the bioautographic technique.

No sparing of B<sub>12</sub> was seen in many experiments with methionine, purine, and pyrimidine mixtures, including acid and alkaline hydrolyzates of deoxyribonucleic and ribonucleic acid, and high concentrations of yeast and plant extracts.

The organism has been deposited in the American Type Culture Collection and the National Collection of Type Cultures.

### Discussion

Aside from the inability of methionine to spare vitamin B<sub>12</sub>, the pattern of the vitamin B<sub>12</sub> requirement of Lochhead No. 38 is at present indistinguishable from that described for higher animals and for *Ochromonas malhamensis*. Newer results with additional B<sub>12</sub>-requiring soil bacteria (5) indicate that the B<sub>12</sub>-pattern of Lochhead 38 is common to several of them. The profusion of natural B<sub>12</sub>-vitamins, some of them inactive for one or another group of microorganisms (5, 7), denotes an extraordinary complexity in their ecology, and a corresponding need for an assay organism with different specificities to distinguish among them. Many of the phytoplanktons at the base of the food pyramid in fresh-water bodies and the ocean need vitamin B<sub>12</sub> (10). The important fisheries of the world are inshore, and even pelagic fisheries are dependent on contacts of the fish with inshore waters (11). The passage from land to sea of members of the vitamin B<sub>12</sub> family of compounds therefore appears to be a promising subject for investigation. A conclusion from the present study is, then, that although the natural history of the B<sub>12</sub>-vitamins is exceedingly complicated, bacterial analytical tools to attack these problems can be found. One wonders whether marine muds contain equivalent bacteria, i.e., having the same kind of vitamin B<sub>12</sub> requirements as the soil isolates, but differing in salt requirements and tolerances, and whether they can be isolated by essentially the Lochhead-Thexton method, suitably modified for marine materials.

The estimation and biochemistry of vitamin B<sub>12</sub> has attracted so much interest, as witnessed by the many reviews, that the present results call for only brief comment; detailed discussion might well await the follow-up of comparative studies of other soil isolates (6). The specificity of the vitamin B<sub>12</sub> requirement in Lochhead 38 emphasizes anew the present ignorance of at least one essential function of the vitamin, for in Lochhead 38, as in *O. malhamensis* and *Euglena gracilis*, it has not yet been possible to bypass the requirement with compounds in whose syntheses the vitamin participates; in this respect, knowledge of vitamin B<sub>12</sub> lags behind that of folic acid.

The ecological specialization of Lochhead 38 is unknown, for there is as yet no enrichment procedure for it. Its utilization of cellobiose and rhamnose suggests trial of plant gums or cell wall materials of bacteria and other microorganisms.

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## APPLICATION OF THE WARBURG RESPIROMETER IN STUDYING RESPIRATORY ACTIVITY IN SOIL<sup>1</sup>

F. E. CHASE AND P. H. H. GRAY

### Abstract

An investigation was made into the feasibility of using the Warburg respirometer to study microbial respiration daily in soil for periods of 1 or more weeks, at levels of temperature and moisture normally occurring in the field. Problems arising from such prolonged continuous operation of the respirometers were overcome; and the method, applied to remoistened air-dried soil, yielded data closely fitting a straight line when log rate of oxygen uptake was plotted against log time (i.e. a parabolic function). The introduction of chemical inhibitors or heat treatment disturbed this linear relationship initially, though it reappeared in many instances, and its reappearance seemed to indicate the resumption of normal microbial activity. Since the parabolic function has no recognizable physiological significance in this application, an hypothesis assuming the simultaneous utilization of substrates of different availability is proposed and discussed.

### Introduction

The use of a modified Barcroft differential respirometer to measure the activity of microorganisms in soil for a 2-day period was described by Smith and Brown (27) in 1932. Since then, little attention has been given to the application of manometric methods to determine the oxidation of organic matter by microorganisms in soil until Webley (33) devised a method using the Warburg apparatus to measure soil aggregation indirectly by determining the rate of diffusion of oxygen into soil, and until Webley, with Quastel (25), reported its application. Quastel and Scholefield (24) also employed the Warburg respirometer in studying the activity of nitrifying organisms. Lees (16) modified a Haldane respirometer and followed the general oxidative activity of the soil population for a week at a time; and more recently, Gamble *et al.* (8) applied respirometry to determine the effect of herbicides on soil organisms. The application of manometric gas analysis involving studies of the ratio of carbon dioxide evolved to oxygen consumed in soil has been reported by two groups of workers (2, 9). None of these investigators has reported using the Warburg respirometer continuously for more than a day or two at a time. This paper gives examples of information that can be obtained concerning respiratory activity of microorganisms in soil when the Warburg respirometer is used for periods extending to a week or more and deals with some special problems that arise in such work. A note introducing the results obtained has preceded this paper (3).

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## Experimental Methods

### *Description of Soils*

Two widely different soils were chosen for these studies, one being muck soil from Macdonald College farm, the other a sandy loam from the Ontario Agricultural College farm. Approximately one cubic foot from the top 6 in. of each soil was obtained in autumn. After air-drying, clods were crushed and the soils screened to remove coarse plant material. The soils were then passed through a 1 mm. sieve to obtain the 1 mm. and smaller fractions for use in these studies. The characteristics of these soils are shown below:

SOIL	MUCK	SANDY LOAM
Condition	Under cultivation	Grass sod (about 20 yr.)
pH	6.9	7.3
Water holding capacity, %	134	39
Organic matter ( $C \times 1.74$ ), %	70	3.9
Specific gravity	1.54	2.67

The soil pH was determined with a Beckman pH meter using the paste method of Reed and Cummings (26); water holding capacity by the procedure of McKibbin and Gray (20); organic matter by the wet combustion method of Walkley (32); and specific gravity according to the method of Lyon and Buckman (19).

### *Method of Using Warburg Respirometer with Soil*

Application of the Warburg respirometer to measure respiratory activity in soil for periods of several days or weeks brought to light difficulties not encountered in conventional experiments of but a few hours duration. Thus it was found that if the water bath was operated 5° C. or more above room temperature for several days so much water vapor condensed in the manometer capillary as to interfere with normal operation. This condensation was usually avoided by operating the bath at 25° C. A 20% solution of potassium hydroxide for carbon dioxide absorption in center wells of Warburg vessels was found unsatisfactory because it extracted moisture from the soil and also tended to creep over the rim of the well after several days, even though the rim had been coated with vaseline (28). The extraction of moisture was slowed considerably by reducing the concentration of potassium hydroxide to a 3% solution, though this necessitated changing the solution at intervals of 2 to 6 days, depending upon the rate of carbon dioxide evolution. The creeping was overcome by using a "paddle-shaped" piece of filter paper in the well, with a "blade" of such width that it touched opposite walls of the well and with a narrow "handle" which extended upwards through the mouth and was arranged carefully so as not to touch the greased rim of the well at any point.

The method of operating the Warburg respirometer was as follows: samples of 1 g. of muck or 2 g. of loam soil were placed in the main chambers of conventional single side-arm Warburg vessels. The soil was distributed evenly on the bottom of the vessels and water was added to adjust moisture to 60% water holding capacity. The rims of the center wells were greased, after

which 0.2 ml. of 3% potassium hydroxide solution was transferred into each well and the "paddle-shaped" pieces of filter paper carefully inserted. The flasks were attached to the manometers in the usual way and the manometers randomized about the water bath so that replicates were well distributed. A thermobarometer consisting of a manometer with its flask containing 2 or 3 ml. of water was included, usually in duplicate. The temperature of the water bath was usually kept at 25° C. and since agitation was not found to give any advantage, the flasks were incubated under static conditions, as proposed by Ellinger and Quastel (7). An equilibration period of 1 hour was allowed the first day and of  $\frac{1}{2}$  hour on each successive day before the stopcocks were closed. Though manometers were read at 2-hour intervals for a 6- to 10-hour period each day, only the first and last of each set of daily readings were used. From these the rate of oxygen uptake characteristic for each day was calculated and expressed in microliters per gram of soil per hour ( $\mu\text{l./g./hr.}$ ). The complete sets of readings were, however, occasionally useful in indicating that a manometer was not functioning properly. As soon as the last reading was made on any day, stopcocks on the manometers were opened, the water circulator was turned off, and the thermostatically controlled heater in the water bath was left on to keep the temperature uniform overnight. The next morning the circulator was started, and after 30 minutes the manometers were closed and respiration measurements started for another day. In this way respiratory activity was followed for as long as four weeks.

#### *Calculation of Flask Constants*

A method of calculating constants of flasks containing soil has been described by Webley (33), whose method was followed except that in these air-dried soils the moisture was so low that it could be disregarded. Webley found he could use the average specific gravity of mineral soils without introducing significant error, but since our experiments involved both a mineral and a muck soil it was necessary to determine the specific gravity of both soils.

#### *Plotting Data*

A few comments on the reports of other investigators will explain the method selected for plotting respiration data. In 1924 Lemmermann and Weissmann (17) first noted the possible existence of a law relating evolution of  $\text{CO}_2$  from organic matter in soil to time. They proposed the equation  $X = a.k.t^m$  where  $X$  equalled total amount of  $\text{CO}_2$  produced in time  $t$ ,  $a$  was the carbon content of the soil at the beginning of the experiment, and  $k$  and  $m$  were constants. They converted this equation to its log form and showed that when  $\log X$  was plotted against  $\log t$  a linear relationship was obtained throughout their experimental period of 995 days; even the addition of hay and straw to the soil disturbed this for only the first 15 to 30 days.

A few years later Corbet (4, 5), studying  $\text{CO}_2$  evolution from Malayan soils, found that the equation applied to his results. He modified the original

equation by combining  $a$  and  $k$  values to give constant  $F$  (fertility constant), which he believed to be an accurate index of the activity of microorganisms in a soil and more pertinent than an enumeration of bacteria by the plate method. He gave the equation as  $Y = Ft^m$  where  $Y$  represented the total yield of  $\text{CO}_2$  from the beginning of the experiment to time  $t$ , and  $F$  and  $m$  were constants. He considered that  $m$ , a measure of the constant rate of decrease of  $\text{CO}_2$  evolution, was dependent upon laboratory conditions. Corbet also noted that the Barcroft manometric data of Smith and Brown (27), while rarely extending beyond two days, conformed to his equation. Millar *et al.* (22), using Corbet's equation, likewise obtained a linear relation between  $\log \text{CO}_2$  and  $\log$  time beginning after the first two weeks and continuing until the experiment was terminated at 280 days.

In our studies, since average daily rates of oxygen uptake or of  $\text{CO}_2$  evolution were calculated more easily than cumulative totals, it was considered advantageous to change Corbet's equation to its rate form, which meant differentiating  $Y$  with respect to  $t$ ; this gave

$$dY/dt = F m t^{m-1}.$$

Now  $F$  and  $m$  being constants, their product would also be a constant; this was designated  $F'$ . It has also been stated that constant  $m$  must be fractional (17), therefore  $m-1$  would be a new constant and become negative; this was represented by  $-m'$ . Incorporating these changes,

$$r = F' t^{-m'},$$

where  $r$  represents respiratory rates at time  $t$ . For use this equation was changed to its log form,

$$\log r = \log F' - m' \log t,$$

and if respiration data yielded a straight line when  $\log$  rate was plotted against  $\log$  time it indicated further support of the mathematical relationship discovered by Lemmermann and Weissmann (17).

Calculations and statistical analyses of  $\log F'$ ,  $\log m'$  constants, and the regression of  $\log$  rate on  $\log$  time were made according to the procedure described by Eisenhart and Wilson (6). Because data obtained on the first day of an experiment were usually low compared with the regression line indicated by later values, such data were not used in calculations, that day being considered an adjustment period.

Early experiments included measurement of both oxygen uptake and carbon dioxide evolution, the latter being determined by the "direct method" (28). It was found that the respiratory quotients were similar for both muck and loam, average values being about 0.65. Since these preliminary studies indicated that oxygen and carbon dioxide curves were essentially parallel, and since oxygen uptake was so much simpler to determine and less subject to variation, carbon dioxide estimations were made only occasionally.

## Results

### *Comparison of Respiratory Activity in Muck and Loam*

A number of experiments could be presented to show this comparison, but the particular experiment selected for purposes of illustration included eight replicates of each soil prepared and incubated simultaneously in a 20 unit Warburg apparatus. The rates of oxygen uptake were determined each day for 11 days. The data presented in Fig. 1 demonstrate the variability among replicates and the regression of log rate against log time. In considering the results from the loam soil no reason could be found to account

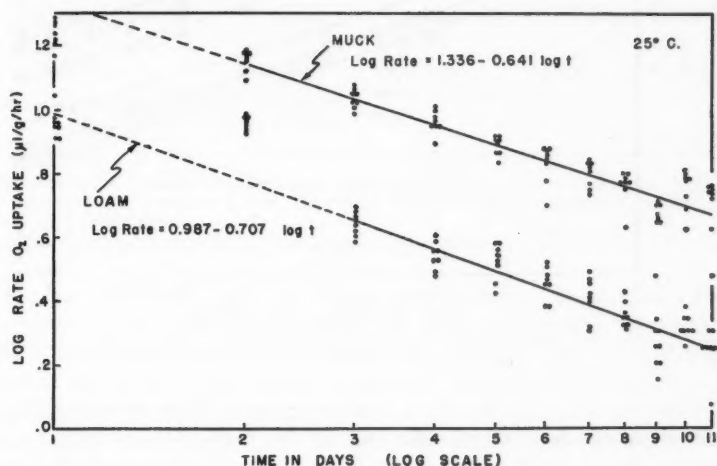


FIG. 1. Regression of log rate  $O_2$  uptake against log time (eight-replicate Warburg respirometer experiment with muck and loam soils).

for the results of the second day being discordant from the rest, so the rates for both the first and the second days were omitted in calculating the regression line. The only values not used in the calculations were first day values from the muck soil.

Points of particular interest are: first, the respiration rate in muck is considerably higher than in loam; and second, the slopes of both lines are very similar—indeed, according to statistical analyses they are not significantly different. In general, however, our studies have shown the slopes for loam to be a little steeper than those for muck, occasionally significantly so; this will be indicated later in Table I. In view of the dissimilarity between the two soils, the similarity of slopes, or  $m'$  values, is particularly interesting and lends support to Corbet's proposal that  $m$  should be considered the laboratory constant since it depends more on laboratory conditions than upon soil characteristics.

### *The Effect of Moisture Level on Respiration in Soil*

A study was made of the effect of varying moisture upon rate of respiration in both muck and loam soils. Moisture levels were varied from 30 to 90% saturation in increments of 15%. Results are presented in Fig. 2. This

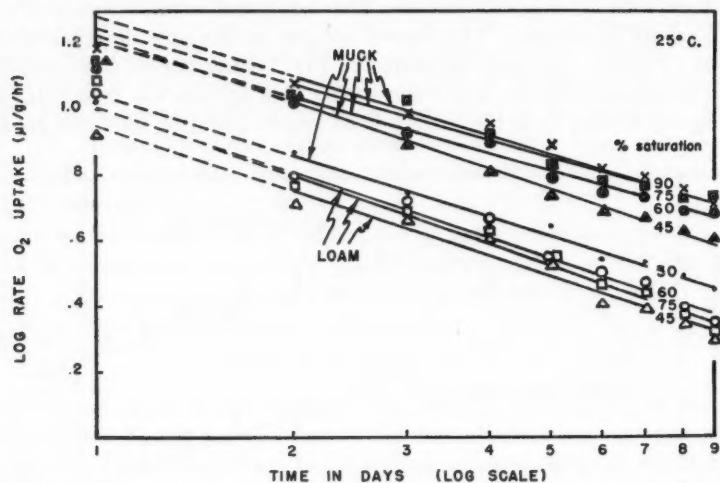


FIG. 2. Effect of moisture level on microbial respiratory activity in muck and loam soils.

graph indicates again, as did Fig. 1, the similarity in slopes of the respiration lines for these two contrasting soils, and, in this instance, even under widely different moisture levels. The results also suggest that in muck the rate of microbial respiration increased with increasing moisture up to the maximum level tested, namely 90% saturation; statistical analysis indicated, however, that only the 30% line was significantly lower than any of the others.

In loam no significant difference in levels of respiration lines appeared for the three moisture levels tested. But it is interesting to note that respiration was consistently highest in the soil adjusted to 60% saturation, the moisture level long accepted as optimum for most microbial activities in mineral soil.

In general these results suggest that the factors studied, soil characteristic and moisture level, exert their greatest influence on the height of the regression line, or  $\log F'$  value, rather than on the slope of the line, or  $m'$  value, when log rate of oxygen uptake is plotted against log time.

### *Effect of Incubation Temperature on Respiration in Soil*

The influence of temperature on soil respiration was examined by collecting the  $m'$  and  $\log F'$  values obtained in 13 experiments involving both loam and muck soils at three incubation temperatures, 25°, 28°, and 30° C. These results are presented in Table I, and indicate a definite tendency for the  $m'$  values to increase with increasing temperature; in other words, the slope of



TABLE I

EFFECT OF TEMPERATURE ON CONSTANTS OF OXYGEN UPTAKE REGRESSION EQUATIONS

Incubation temperature	Loam		Muck	
	Log $F'$	$m'$	Log $F'$	$m'$
25° C.	1.040	0.694	1.093	0.587
	1.044	0.665	1.222	0.672
	0.987	0.707	1.208	0.651
			1.204	0.561
			1.336	0.641
			1.316	0.646
28° C.	1.010	0.809		
	1.104	0.820		
30° C.	1.074	0.848	1.438	0.962

L.S.D. (5% point) log  $F'$  0.092\*  $\pm$  0.002†  
 $m'$  0.101\*  $\pm$  0.002†

\*Based on error variance obtained from eight-replicate series (considered best estimate of error variance) and given as L.S.D. for 10-day duplicate-flask experiments, which include most tabular comparisons.

†Largest correction required for any comparison in table.

the line becomes steeper with increasing temperature. This appears logical, since elevating the temperature within the range used would be expected to increase the rate of microbial oxidation (2), which in turn should result in an earlier depletion of soil organic matter and become manifest on the graph as a steeper slope of the respiration line.

The log  $F'$  values, which express the theoretical respiration rate for the first day of an experiment, would also be expected to increase with an increase in temperature. This occurred with the muck soil, but in the loam, although the average log  $F'$  values did increase as temperature was raised, the increases were not statistically significant.

In connection with the log  $F'$  values obtained for the muck soil incubated at 25° C., it is interesting to note in reading down the column that there is a progression from low to high values. As these values have been placed in chronological order and cover a storage period of the dry soil sample extending to 12 months, it seems that this soil may be acting similarly to some described by other workers (21, 31) who found that the amount of carbon dioxide evolved during the first few days after remoistening increased with the length of storage. Such an effect was not apparent with the loam.

#### *Response of Soil Respiration to the Addition of Chemical Inhibitors*

The effect of chloretone upon soil respiration was determined by adding water containing 0.4% chloretone to air-dried soil in Warburg vessels in sufficient quantity to adjust moisture to 60% saturation. In muck soil the chemical apparently stimulated respiration, since the regression line was slightly raised and the slope left unchanged. Even the use of a 0.8% solution

of chloretone showed a stimulating rather than inhibiting effect after the first two days, the regression line being parallel to that of the control soil and a little higher than that obtained with the lower concentration.

The loam showed altogether different results. Only the 0.4% aqueous solution of chloretone was used. The results are presented in Fig. 3, with each point representing the mean of duplicate determinations of oxygen uptake.

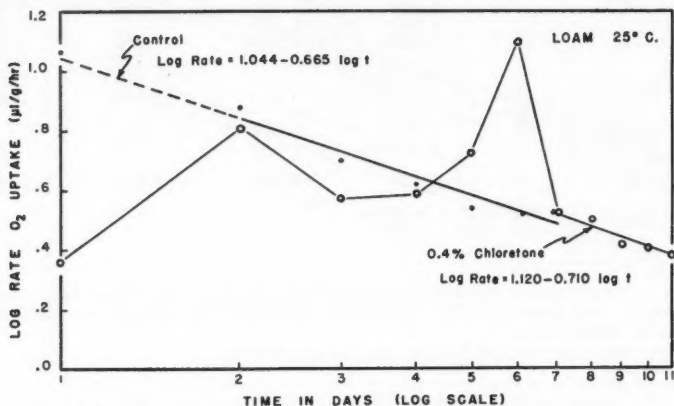


FIG. 3. Effect of chloretone on rate of  $O_2$  uptake in loam.

It is apparent that the chloretone treatment retarded respiration for the first four days, following which on the fifth and sixth days the rates of oxygen uptake greatly exceeded that in the control series. Apparently an adaptation had occurred and the inhibitory effect of the chloretone was overcome. A very interesting development occurred, however, on the seventh day when the linear log rate - log time function appeared with the slope similar to that of the control but at a slightly higher level. One is tempted to conclude that by the seventh day the impact of the chloretone treatment on the activity of the soil organisms has been completely overcome, since the characteristic respiration slope is resumed at this time.

The difference in reaction to chloretone treatment between the two soils seems likely to be due to the protective action of the greater amount of organic matter in the muck soil.

Another interesting application of the respiratory method of studying the activities of soil organisms was made by the determination of both oxygen consumption and carbon dioxide evolution following treatment of the loam soil with mercuric chloride. This experiment was made because of anomalous results obtained in preliminary trials in which carbon dioxide evolution (determined by the aeration method) was used as the index of the degree of inhibition by various chemical agents. It had been found that the addition of mercuric chloride to the loam soil resulted in a marked evolution of carbon dioxide, even though plate counts indicated destruction of almost

all the microorganisms. Since the mercuric chloride treatment was found to have reduced the pH, and since the loam contained free carbonate, it was suspected that the carbon dioxide was produced by chemical rather than biological action. As the Warburg respirometer can be used to measure both oxygen uptake and carbon dioxide evolution, it appeared to be logical to use the apparatus in searching for the source of this carbon dioxide. The results of an experiment in which both oxygen uptake and carbon dioxide evolution were measured from the loam with and without mercuric chloride applications are shown in Table II. It is apparent that the addition of

TABLE II

EFFECT OF MERCURIC CHLORIDE ON CARBON DIOXIDE EVOLUTION AND OXYGEN UPTAKE IN LOAM

Mercuric chloride ( $\mu\text{g./g. soil}$ )	Gas exchange	Rate of gas exchange measured daily ( $\mu\text{l./g./hr.}$ )						
		1	2	3	4	5	6	7
0	$\text{CO}_2$	6.7	4.4	3.0	2.2	1.9	2.1	2.6
	$\text{O}_2$	11.7	7.6	5.2	4.2	3.5	3.4	3.4
	RQ	0.57	0.58	0.58	0.52	0.54	0.62	0.76
14	$\text{CO}_2$	13.5	3.4	1.8	1.5	1.2	1.2	1.0
	$\text{O}_2$	3.0	1.4	0.8	0.6	0.6	0.6	0.4
	RQ	4.50	2.42	2.25	2.50	2.00	2.00	2.50
32	$\text{CO}_2$	14.0	5.5	3.1	2.3			
	$\text{O}_2$	0.5	0.0	0.0	0.0			
	RQ	28.0						

mercuric chloride resulted in a marked deviation from the normal relationship between oxygen uptake and carbon dioxide evolution as revealed by the control units. Evolution of carbon dioxide was doubled on the first day by the mercuric chloride treatment, following which carbon dioxide production continued at daily rates not unlike those in the control. Oxygen uptake was almost stopped by the lower concentration of mercuric chloride and was apparently completely blocked at a higher level. This indicates that the carbon dioxide produced after mercuric chloride treatment probably arose from chemical rather than biological action. These results also show that the measurement of oxygen consumption rather than carbon dioxide evolution may possess a marked advantage in certain soils. However, careful interpretation during such studies seems necessary, since Bunt and Rovira (2) have reported experiments with Australian soils where the oxygen consumption was largely accounted for by chemical oxidation.

#### *Effect of Heat Treatment on Oxygen Uptake in Muck Soil*

In this experiment 2 g. of muck soil was used in each flask. The plan called for four replications of four treatments, the latter being as follows: first, 2.00 g. untreated soil; second, 0.67 g. autoclaved plus 1.33 g. untreated soil; third, 1.33 g. autoclaved plus 0.67 g. untreated soil; and fourth, 2.00 g.

autoclaved soil. The required amounts of soil were placed in Warburg flasks, which were plugged with cotton and autoclaved at 15 lb. for 30 minutes. As the soil was not moistened it was not expected that all organisms would be killed; the object was to obtain partial sterilization rather than complete sterility. The results are presented graphically in Fig. 4. The most interest-

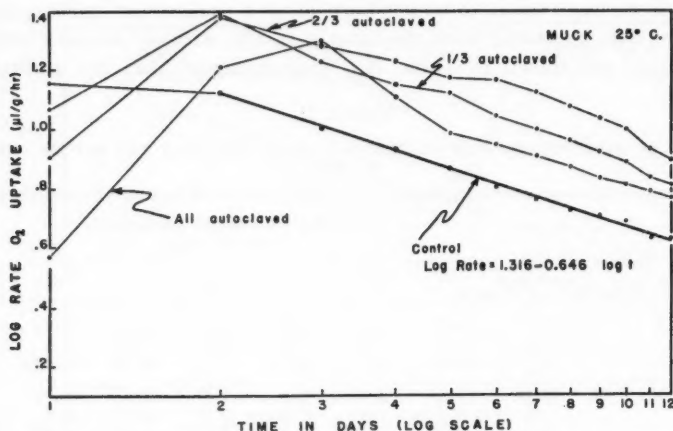


FIG. 4. Effect of autoclaving varying proportions of air-dried muck soil on daily rates of  $O_2$  uptake obtained after remoistening the soil.

ing feature is that in spite of heat treatment, the slopes, after adjustment periods of varying lengths of time, resemble those of the control, though the general level has been increased. Thus the increase in respiratory activity following heat treatment reported by others using carbon dioxide evolution as the indicator (30, 31) has been demonstrated in respirometer studies by measurement of oxygen uptake. Also the graph indicates that by increasing the proportions of heat treated soil, the oxygen uptake was further depressed on the first day. This reaction is quite different from that reported by Bunt and Rovira (2), working with Australian soils. However, the heat treatments they employed were more drastic than those used during our studies.

It is tempting, on the basis of experience to date, to consider the slope of the control soil "normal" and thence to conclude that the flasks containing one-third and two-thirds autoclaved soil had completed the adjustment period by the second or third day while those in which all the soil was heated did not return to "normal" until the fifth day. This demarcation between an adjustment period and the resumption of the normal slope was noted in the experiment with chloreton. It seems that this plotting method may be helpful, in some instances, in determining how long it takes the soil microflora to adapt itself to altered conditions or to recover from the effects of some agent whether harmful or otherwise.

### Discussion

One of the outstanding features evident during this application of the Warburg respirometer to studies of decomposition of soil organic matter has been the reproducibility of the results under controlled conditions. This has encouraged the authors to have considerable confidence in the method. Whether or not such confidence will be justified can only be discovered by continued study. However, an effort to ascertain just how wide an application these oxygen uptake methods may have seems highly desirable, because in soils where it proves dependable the time needed to obtain an estimate of microbial activity might be shortened considerably, since adherence of the calculated results to a straight line function would make it possible to predict later values once the starting point and slope were established.

In the present study, after physical or chemical treatments were applied to injure the soil microorganisms a period of adaptation and redevelopment has been clearly indicated by oxygen uptake rates, following which a definite transition to a log rate - log time function similar to that in untreated soil occurred.

By far the most intriguing question arising from these studies was, in our opinion, the significance or meaning of the linear log rate - log time function. It was obvious, after this straight line relationship appeared in several experiments, that the respiratory activity of the soil organisms was of a constant uniform nature. An attempt was therefore made to consider theoretical aspects of the decomposition of soil organic matter. Lemmermann and Weissmann (17) reported that carbon dioxide evolution from soil did not follow either the first- or second-order reaction law; but they did note the similarity of their equation to Schütz law, which relates the amount of substrate transformed to the square root of time. Moelwyn-Hughes (23) discusses this law and indicates that, in all probability, the controlling factor is rate of diffusion. However, since this law would require that the  $m$  constant always equals 0.5, a condition which certainly is not fulfilled, this law obviously does not apply in these studies.

An attempt was then made to see if the discoveries of various investigators could be related in any way to give a plausible explanation of the linear log rate - log time function. Many workers have found that when an air-dried soil is remoistened an unusually rapid production of  $\text{CO}_2$  occurs the first day or so (1, 10, 21, 29). Others, studying the factors responsible for this stimulation of microbial activity, have shown that drying renders the organic matter more readily decomposable; and this has been further substantiated by recorded increases of water-soluble organic matter after the drying of soil (11, 14, 15). From this we can conclude that immediately after a dried soil has been remoistened there will be present, in addition to the more slowly decomposable humus complex, a limited supply of readily available water-soluble organic matter. If we now introduce Winogradsky's idea that the soil organisms are divided into two major groups, namely, the indigenous microflora concerned with the slow breakdown of the humus, and the zymo-

genous microflora which rapidly attacks any readily fermentable substance (34), we have the basis of an interesting hypothesis. Let us suppose that these two groups of organisms are functioning independently, each upon its selected substrate according to the exponential or first-order reaction law, with the concentration of substrate being the limiting factor. We would then have not one but at least two different first-order reactions superimposed one upon the other, with the result that the combined values, if plotted log rate against linear time, could not be expected to yield a straight line, but rather a curve with at least two segments.

The data from a number of our experiments were examined by plotting the logarithms of the rates of oxygen uptake against time; they showed a definite tendency to give a graph with an initial curve followed by a straight line. This is illustrated in Fig. 5. By assuming that the second, or straight line

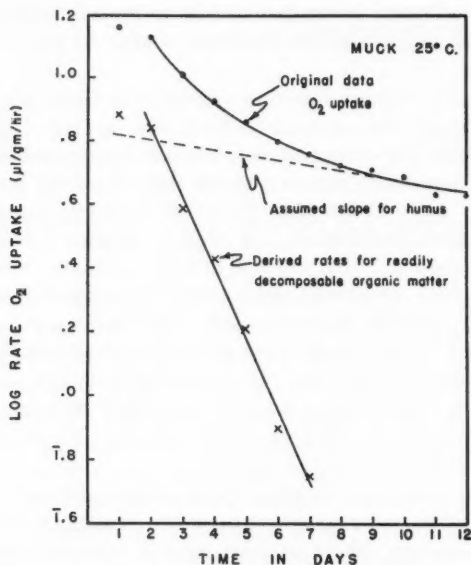


FIG. 5. Data from control series (Fig. 4) analyzed on assumption of two superimposed first-order reactions.

portion of the curve is due to the breakdown of humus, this humus line may then be projected backwards, on the premise that humus was being broken down in accordance with this straight line function since the start of the experiment. It is then possible to calculate, by difference, the rate of oxygen uptake accountable each day for the oxidation of the more readily fermentable organic fraction. When the logarithms of these daily rates were plotted against time a reasonable approximation to a second straight line was obtained.



These results support the hypothesis, presented previously by us in outline form (3), that the log rate - log time straight line function, which seems to apply empirically to soil respiration data, may arise from a situation where two or more first-order reactions are progressing simultaneously. It is recognized that there are probably a number of components of native soil organic matter which conceivably serve as substrates in a whole series of superimposed first-order reactions, each with distinctive reaction velocities. The fact that the soil organic matter appears to be made up of a simple two-component complex suggests that the reaction velocities of two types of substrate (which we might designate as "readily available" and "humus-A") are large in comparison with the residuals which may be expected to appear only after the more easily oxidizable fractions have been partially depleted.

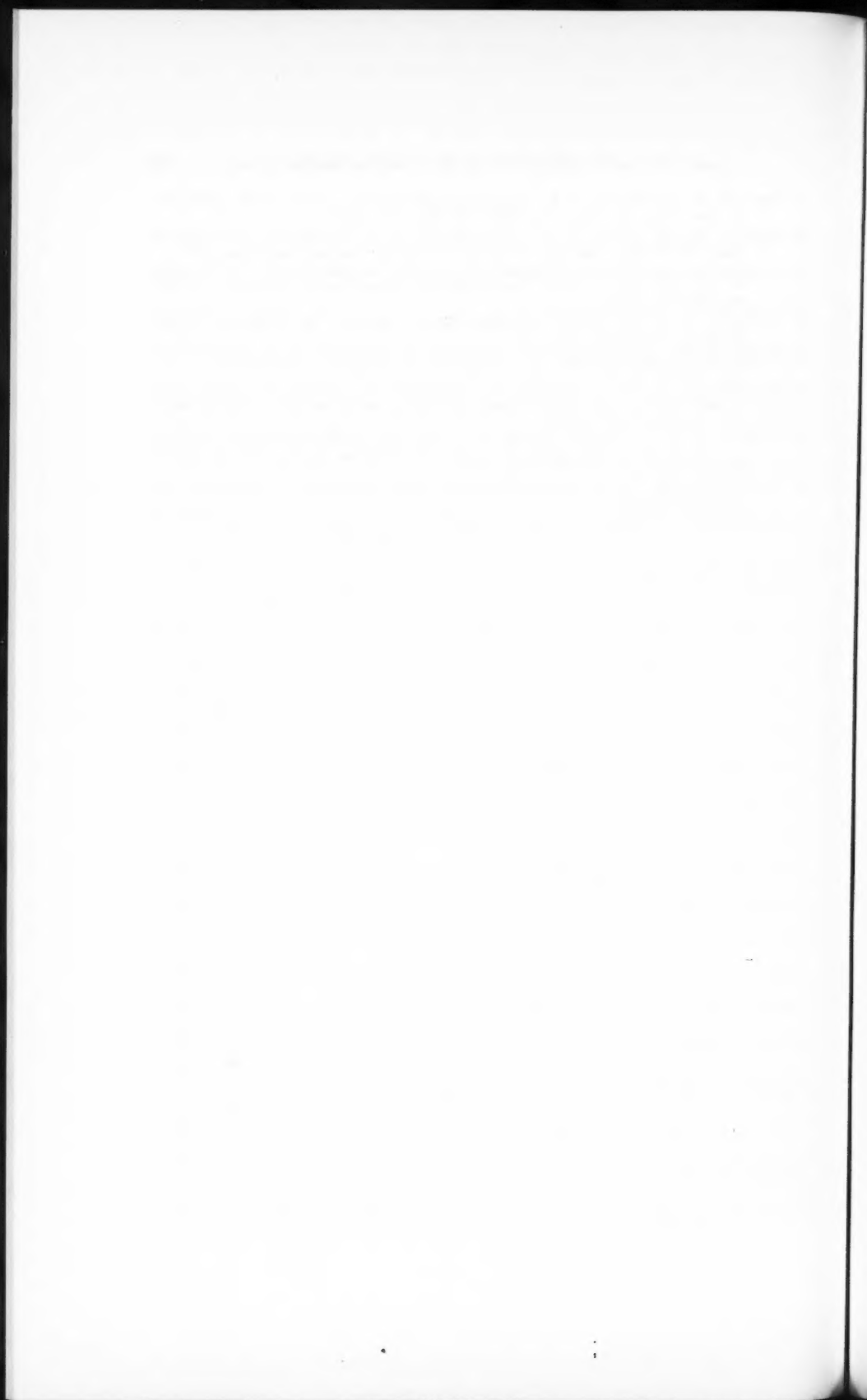
A similar mathematical treatment, developed independently in an entirely different field, has recently come to our attention. Lietzke (18), using world records established in many racing events, showed that a straight line was obtained by plotting log distance against log time; this involved use of the same general equation employed by Corbet in his soil respiration studies (5). Lietzke also presented a log rate - log time plotting that corresponds to our conversion of Corbet's equation to its rate form in the present investigation. More recently, Henry (12, 13) pointed out that Lietzke's plotting method (really based on the parabolic function with no known physiological rationale) was empirical and proposed, as a more fitting basis for study, the hypothesis that "maximum speed in running is limited by the energy reserves available for conversion into work, and that each of these resources begins to be depleted from the very beginning of the race in accord with an exponential law" (12). This expresses, in connection with world record running events, essentially the same idea of superimposed first-order reactions as we discussed above in connection with the microbial oxidation of soil organic matter.

The division of respiratory activity into two first-order reactions, as shown in Fig. 5, permits further deductions as to reaction rates. Thus it is possible to ascertain, from the slopes of the lines, the half-life times of the two substrates, which prove to be 1.4 days and 15.6 days respectively for "readily decomposable" and "humus-A" materials. The term "humus-A" is used here in recognition of the fact that soil humus (excluding the readily decomposable water soluble organic fraction that accumulates during the air-drying of soils) comprises many organic substances which probably range through a series of gradations from "moderately available" for microbial oxidation at one extreme to "very resistant" at the other, and that we are dealing primarily with the "moderately available" humus in this instance. The derivation of rates for the more resistant forms of humus will require longer term experiments and probably methods different from those reported here.

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## MICROBIOLOGICAL STUDIES ON AN ORCHARD SOIL UNDER THREE CULTURAL PRACTICES<sup>1</sup>

I. L. STEVENSON<sup>2</sup> AND F. E. CHASE

### Abstract

Respiration studies using aeration and manometric techniques have shown that maximum respiratory activity occurred in mulched soil, followed in descending order by sod and 'clean cultivation: buckwheat cover-crop' methods of soil management. Less effect was noted in cellulose decomposition studies, though the fact that the decomposition of added cellulose began with the shortest lag period in the mulched soil suggests that this treatment maintained cellulose decomposing organisms at the highest level. Enumeration of bacteria, fungi, and actinomycetes at monthly intervals over a period of 15 months revealed only minor differences due to the mulch, sod, or clean cultivation treatments. Distinct seasonal fluctuations in numbers of bacteria and fungi were noted.

### Introduction

During studies of methods of orchard management at the Ontario Horticultural Experiment Station, it was noted that peach trees grown under mulch treatment exhibited a better physical appearance and produced larger yields of better quality fruit than trees grown under other methods of orchard management (15). The effect of mulching on the soil was to improve its structure and increase both the organic content and the water holding capacity. Similar results have been described by other workers studying the effects of organic mulches on the physical and chemical properties of soils (4, 5, 13). Since the effect of mulching produced a greater response than could be attributed to the mulch as a source of inorganic nutrient, it was felt that a study of the soil microbial processes might aid in the understanding of the action of this material.

Numerous microbiological methods have been described in the literature for the assessment of soil fertility. Waksman (16) recorded a few instances in which bacterial numbers and crop yield have been correlated. However the value of enumeration procedures is known to be limited by such factors as sampling error and the normal fluctuations that occur in the soil population. Some success has been attained in the determination of microbiological activity through methods designed to measure the accumulated by-products of microbial action. Thus, carbon dioxide evolution, cellulose decomposing power, and the nitrifying capacities of soils have all received considerable attention (17, 18, 19). An extensive study of the relationship between microbiological activity and soil fertility was made by White *et al.* (20), who obtained highly significant correlations between crop yields and microbiological activity in soils as measured by soil respiration, cellulose

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decomposing power, and nitrifying capacity. They concluded that the activities of the soil microorganisms may serve as indices of fertility.

This paper deals with an investigation of the microbiological activity in an orchard soil under three systems of cultural management. Because of the difficulties involved in estimating numbers of soil microorganisms, this phase of the study was limited to monthly counts. Emphasis was placed on carbon dioxide evolution, oxygen uptake, and cellulose decomposing power as measures of microbial activity. The effect of these same three systems of cultural management on nitrifying power in this soil has been reported previously (14).

### Materials and Methods

#### *Description of Orchard*

This study was made on a peach orchard located at the Ontario Horticultural Experimental Station, Vineland, Ontario. The soil has been classified as a Vineland fine sandy loam, which is low in lime content and consequently tends to be acid in reaction. The area was divided into three plots which had been under the following methods of orchard management for a period of 4 years prior to the start of this investigation:

- I. Clean cultivation during fall and spring with one buckwheat cover crop grown and incorporated into the soil each year. Soil pH 5.0-5.5.
- II. Grass sod, mainly orchard grass with some Canada blue grass. Soil pH 4.2-4.7.
- III. The same type of grass sod plus annual applications of mulch around the trees. The first mulch consisted of straw, the second and third of hay, mown from between the trees, and the fourth consisted of a 4 in. layer of finely chopped spruce bark containing some poplar sawdust. Soil pH under the mulch was 5.7-5.9.

Each plot consisted of two rows of 12 trees each.

All soil samples were taken to a depth of 4 to 5 in. using a Lamotte sampling tube. Composite samples consisted of cores removed from three positions around all trees in a treatment plot. In the mulch treatment the organic layer was lifted from the soil surface before the samples were collected.

#### *Carbon Dioxide Evolution and Cellulose Decomposition*

The aeration method described by McKibbin and Gray (9), modified by removing carbon dioxide from the air before passage over the soil, was used in these studies. Daily evolution of carbon dioxide is expressed as  $\mu\text{l. CO}_2/\text{g. soil/hr.}$ , and respiratory capacity as total  $\mu\text{g. CO}_2$  evolved per g. of air-dried soil in 14 days.

In the determination of the cellulose decomposing power by this technique paired units were used, one containing soil alone and the other containing soil plus 1% cellulose added as ground Whatman No. 1 filter paper. The cellulose decomposing power was determined from the difference in amounts of carbon dioxide evolved in the paired units and is recorded as total  $\mu\text{g. CO}_2$  evolved per g. of soil in 14 days.



*Manometric Determination of Oxygen Uptake and Cellulose Decomposition*

Microbial respiratory activity was also determined by measuring oxygen uptake by the manometric method described by Chase and Gray (1). Rates of oxygen uptake characteristic for each day of a 2-week experimental period were determined and recorded as  $\mu\text{l. O}_2/\text{g. air-dried soil/hr.}$  As in the aeration method paired flasks were required for the manometric determination of cellulose decomposition. Differences in daily rates of oxygen uptake between a flask containing soil plus 1% cellulose and one containing soil alone were assumed to be due to cellulose decomposition and expressed as total  $\mu\text{g. O}_2$  per g. air-dried soil in 14 days.

*Plating Methods*

Total numbers of bacteria and actinomycetes were determined by plating suitable dilutions of fresh composite soil samples with soil extract agar. Plates were poured in quadruplicate and incubated for 10 days at  $26^\circ\text{C.}$  Counts of fungi were made using the rose bengal medium of Smith and Dawson (12). Plates were incubated for 5 days at  $26^\circ\text{C.}$

The average numbers of colonies on the replicate plates were corrected for the moisture content of the soil and recorded as colonies per gram of oven-dried soil.

## Results and Discussion

*Respiration Studies*

Curves indicating the carbon dioxide evolution that occurred in composite samples of the plots under each of the three soil treatments with and without added cellulose are shown in Fig. 1. In all untreated soil, maximal carbon dioxide evolution was obtained on the 1st day and was followed by a gradual decrease until the end of the 14-day period. This initial burst of carbon dioxide that occurs when an air-dried soil is remoistened has been noted by other investigators (11), and probably arises from the microbial decomposition of soluble organic matter that accumulated during the air-drying period (1, 7).

Cellulose decomposition curves show the existence of a 2-day lag period during which respiration rates were the same as those in the soil without added cellulose. Maximal rates of carbon dioxide evolution due to cellulose utilization were reached between the 4th and 7th days, after which there was a gradual decrease until the end of the experimental period.

The respiratory capacities and the cellulose decomposing powers of the soil under the three treatments, as indicated by carbon dioxide evolution, are presented in abridged form in Table I. Statistical analyses of the complete data showed a significantly greater respiratory activity in the mulched soil than in samples from the sod, and the sod treatment gave a significantly higher respiratory capacity than that obtained from clean cultivated treatment. No significant differences in the cellulose decomposing power occurred under any of the treatments.

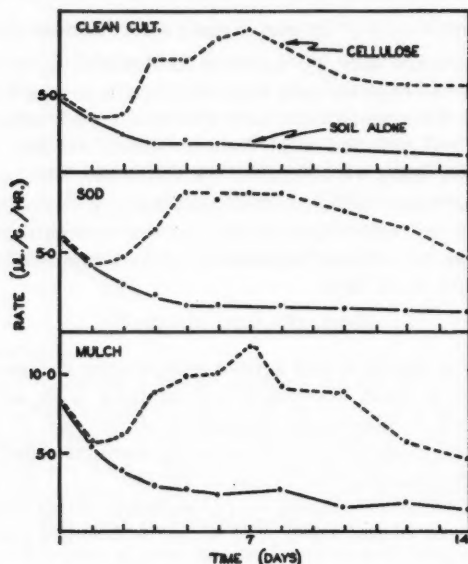


FIG. 1. Daily rates of carbon dioxide evolution of soil alone and soil plus cellulose.

Respiration data obtained using manometric methods are given in Table II. Examination of the data shows that the respiratory capacities of the soils under the three methods of management, when placed in descending rank, followed the order: mulch, sod, and clean cultivation treatments. Results by the manometric method also indicated that the cellulose decomposing power was not significantly different under any of the cultural practices. Daily rates of oxygen uptake, when plotted against time, yielded curves similar to those for rates of carbon dioxide evolution shown in Fig. 1, though smoother in outline. Thus the aeration and manometric methods were in agreement.

Although the manometric data indicated that none of the cultural practices had significantly affected the cellulose decomposing power of the soil, certain differences were noted when the logarithms of oxygen uptake rates were plotted for the first 7 days of the experiment. Two features are noteworthy in the curves shown in Fig. 2; first, the exponential increase in rates of cellulose decomposition—until maximal activity was reached about the end of the 1st week; and second, the appreciable differences in length of the lag periods. The exponential increase in respiration rates of the cellulose decomposing organisms, particularly in the sod and the clean cultivation treatments, is similar to what would be expected in pure culture studies during the logarithmic phase of increase of the bacterial growth curve. It appears that this plotting method has demonstrated the exponential increase of the cellulose decomposing microorganisms despite the presence of an actively respiring heterogeneous microbial population in soil. The obvious differences in length of lag periods indicated by this plotting procedure are also of interest.

TABLE I

TOTAL CARBON DIOXIDE EVOLUTION DUE TO SOIL RESPIRATION AND TO CELLULOSE DECOMPOSITION UNDER THE THREE TREATMENTS FOR SERIES I AND II  
( $\mu\text{g. CO}_2$  per g. air-dried soil in 14 days)

	Clean cultivation		Sod		Mulch	
	I*	II	I	II	I	II
Soil alone	1240†	1450	1380	1750	1830	2360
Cellulose decomposition	2310	2240	2720	2360	2670	2090

\*Series I and II based on plot samplings (and analyses) made 14 months apart.

†Each table entry is the average of duplicate determinations.

TABLE II

TOTAL OXYGEN UPTAKE DUE TO SOIL RESPIRATION AND TO CELLULOSE DECOMPOSITION UNDER THE THREE TREATMENTS FOR REPLICATES A AND B  
( $\mu\text{g. O}_2$  uptake per g. air-dried soil in 14 days)

	Clean cultivation		Sod		Mulch	
	A	B	A	B	A	B
Soil alone	645	627	674	661	1056	1015
Cellulose decomposition	1783	1806	2514	—	1926	1835

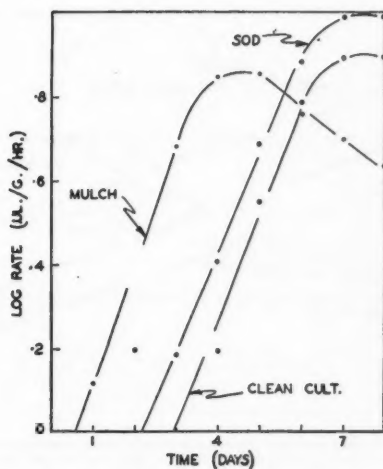


FIG. 2. Daily rates of oxygen uptake due to the addition of cellulose.

Although the cellulose decomposing power (as indicated by the total oxygen uptake due to cellulose decomposition occurring in a 2-week period) was apparently not significantly affected by any of the three management practices, yet it seems logical to expect that mulching should bring about some increase in the activity of cellulose decomposing organisms; the fact that the shortest lag occurred in the mulched soil indicates that such must have been the case. Also as the longest lag period was associated with the clean cultivation treatment, it was apparently the poorest in maintaining an active population of cellulose decomposing organisms; this likewise appears logical. These results indicate that the manometric method combined with this plotting procedure may be of value in studying the activity of cellulose decomposing organisms in the soil under various treatments.

Attempts have been made from time to time to determine whether a stable mathematical relationship exists between respiratory activity in soil and time (1, 2, 8). In the most recent of these, Chase and Gray (1) proposed the following equation:

$$\log r = \log F' - m' \log t$$

where  $r$  represents respiratory rate at time  $t$ , and  $F'$  and  $m'$  are constants. Fig. 3 displays typical regression lines obtained by using this equation to analyze data from an experiment in which oxygen uptake rates were determined each day for a week in soil samples taken from each of the three cultural practices; each point on the graph represents the mean of quadruplicate determinations. The regression lines show no appreciable difference in the slopes ( $m'$  values), which seem to depend primarily on conditions prevailing in the laboratory (1, 2), but definite differences are apparent between the ordinate intercepts ( $F'$  values) of the regression lines for each of the soil

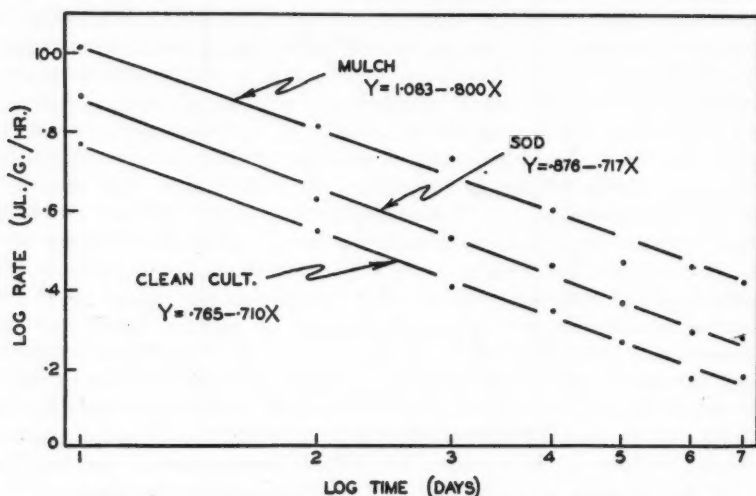


FIG. 3. Typical regression lines for oxygen uptake of soil alone.

treatments. These  $F'$  values are related to Corbet's 'Fertility' constant (2). The mulch treatment clearly shows the highest level of respiration and is followed by the sod and clean cultivation treatments in descending order. The constancy with which the respiration data from each of the treated orchard soils follow the corresponding regression lines supports the suggestion of previous workers (1, 2, 8) that the microbial flora attacks the residual organic components of soil in a definite manner which can be expressed mathematically.

#### *Numbers of Bacteria, Actinomycetes, and Fungi*

Counts of bacteria, actinomycetes, and fungi were made at monthly intervals for 15 months; 1 month was missed when weather conditions prevented sampling. The results, presented in Fig. 4, indicate that there was a marked effect of season upon bacterial numbers, a definite seasonal change in numbers of fungi, and a slight effect of season upon counts of actinomycetes. These results are in agreement with those of numerous investigators who have shown that bacteria (3, 10), actinomycetes (3), and fungi (6) are all susceptible to seasonal fluctuations. Of the three groups, only the actinomycete population showed any stability in response to the different methods of orchard management; mulching proved most favorable to the actinomycetes, with clean cultivation being intermediate and the sod treatment lowest during the 15-month period.

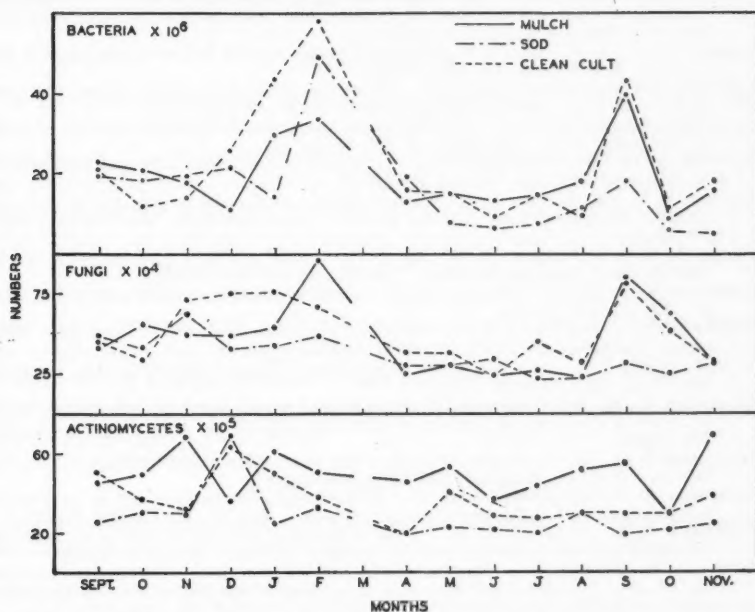


FIG. 4. Numbers of bacteria, fungi, and actinomycetes at monthly intervals.

While these treatments showed no uniform effect upon bacterial numbers, mulching did apparently exert a stabilizing influence. The mulch treatment was more favorable than either of the other treatments for the bacterial population during the summer and yet was characterized by the lowest plate counts during most of the winter. It is felt that the numerous freezings and thawings during the winter months increased the plate counts of bacteria in the clean, cultivated soil and that this effect was less noticeable in the mulch- and sod-treated soils because they were protected more from temperature extremes by the nature of their cover. The favorable effect of mulch during the summer on the bacterial population is probably due in part to the moisture conserving effect of this treatment, for it was observed that while soil from the other treatments became dry during the summer, samples from beneath the mulch were always moist.

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## NITROGEN LOSSES DURING NITRIFICATION IN SOLUTIONS AND IN ACID SANDY SOILS<sup>1</sup>

F. C. GERRETSEN AND H. DE HOOP

### Abstract

Soil nitrogen balances involving lysimeter experiments and cropped and uncropped pot experiments have shown that in many cases such important nitrogen deficits were observed that there must be hitherto unknown or unsuspected pathways along which nitrogen escapes. In former experiments of the senior author it was shown that in acid soils, dressed with ammonium sulphate, nitrogen can be lost as N—O compounds during nitrification. In the present investigation more accurate determinations made it clear that when the pH of a liquid culture of nitrifying bacteria dropped below about 5.5 nitrogen was not only lost as N—O compounds, but to a greater extent as nitrogen gas, most probably by chemical reaction between the  $\text{HNO}_2$  formed during nitrification and the ammonia present in the solution. In pot experiments with acid sandy soils from different parts of the Netherlands, losses of up to 74% of the ammonium sulphate added were observed. An investigation was made of the conditions which promote or are essential for these losses. When the initial pH of the culture medium, be it a solution or soil, enables the nitrifying bacteria to develop and the buffer capacity is of such a magnitude that the pH drops below 5.5 during nitrification, volatilization is to be expected. As volatilization and ammonification often coincided, both processes seem to be linked in some way or another; volatilization, however, has also been observed in the absence of ammonification. These losses are not caused by evaporation of ammonia, in which case they should increase as the pH increases; the contrary happens, however, and the losses stop entirely when sufficient  $\text{CaCO}_3$  is added to keep the pH above 5.5. They are also not due to denitrification, as they do not occur when nitrogen is added as nitrate instead of ammonium sulphate. Also there are no losses when the soil is pasteurized, which proves that they originate in bacteriological processes.

### Introduction

In previous planted and unplanted pot experiments (4, 5) it was shown that in two of four acid sandy soils (pH 4.5–5.4) considerable amounts of nitrogen, varying from 10 to 78% of the ammonium sulphate added, were lost most probably as volatile N—O compounds. In a recent review on soil nitrogen balances, Allison (1) concluded that substantial losses occurred in a large number of lysimeter experiments, varying from an average of 20% of the total available nitrogen for cropped soils to 12% for uncropped soils. According to Allison "these data constitute strong evidence that nitrogen losses from normal, well aerated soils by *volatilization* are not negligible". Where this is the case it seemed important to verify the results of our previous experiments on a larger number of soils in order to be better informed on the cause of these nitrogen losses and to investigate in more detail the circumstances under which they occur.

### Experimental Methods

As the nitrogen losses probably occur during nitrification, nitrifying bacteria were cultivated in an aerated culture solution which contained per liter: 1 g.

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Contribution from the Agricultural Experiment Station and Institute for Soil Research T.N.O., Groningen, Netherlands.

$(\text{NH}_4)_2\text{SO}_4$ , 2 g.  $\text{KH}_2\text{PO}_4$ , 1 g.  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 g.  $\text{NaCl}$ , 0.05 g.  $\text{FeSO}_4$ , 0.01 g.  $\text{MnSO}_4$ , and 0.5 g.  $\text{CaSO}_4$ . The pH of the solution was fixed at 6.5 by adding a few milliliters of a 10%  $\text{Na}_2\text{CO}_3$  solution to this medium. The culture solution was inoculated with a few grams of a garden soil, and after the ammonium had disappeared a few milliliters of the medium were transferred to a fresh culture solution and the procedure was repeated until a rather vigorous nitrifying culture was obtained. It is evident that this was not a pure culture, but for our purpose this was not necessary.

Our first experiments were carried out in Erlenmeyer flasks of  $1\frac{1}{2}$  liters, containing 1 liter of culture solution. They were provided with an air inlet tube fitted with a small aquarium aeration stone, an air outlet tube, a glass electrode, and a saturated calomel electrode. Ammonia nitrogen and  $\text{NO}_2^- - \text{N} + \text{NO}_3^- - \text{N}$  were determined before and after the experiment.

In a second series the air that had passed through the culture solution was scrubbed through a glass tube, 30 mm. inside diameter and 1 meter long, filled with pumice stones drenched in a permanganate solution, containing 8 g.  $\text{KMnO}_4$ /liter 30%  $\text{NaOH}$ , to absorb the  $\text{NO}_2$ . A similar tube was used to absorb any  $\text{NO}_2$  from the air before it entered the culture solution. As in this way the air was deprived of  $\text{CO}_2$ , it was passed through a 10%  $\text{NaHCO}_3$  solution adjusted to a pH of about 5.8; this solution was renewed daily. At the end of the experiment the total nitrogen of the contents of the whole absorption tube was determined as well as the  $\text{NH}_4^+$ ,  $\text{NO}_2^-$ , and  $\text{NO}_3^-$  content of the culture solution.

In order to investigate the occurrence of nitrogen losses in soils, a dozen acid sandy soil samples of 5 kg. each from different parts of the country were dressed with 400 mg. N as ammonium sulphate per kilogram. Part of the pots received  $\text{CaCO}_3$  in quantities which depended on the acidity and buffer capacity of the soil. The Mitscherlich pots were stored at  $24^\circ \text{C}$ . during 3 months and at intervals of 3 weeks the ammonia, nitrite, and nitrate N were determined. As the nitrogen determinations had to be very accurate, special attention was paid to homogenizing the soil by repeated sieving and mixing. In a few cases the pots were planted with *Brassica napus* and in these cases the total nitrogen balance also included the nitrogen assimilated by the plants. Different characteristics of a number of soils have been determined, viz. granular composition, total carbon, carbon in humic substances, total nitrogen, nitrogen in humic substances, pH, and buffer capacity towards  $\text{CaCO}_3$ . It was shown, however, that in most cases these characteristics had no relation to the nitrogen losses at all, pH and buffer capacity excepted. In order not to complicate this report, these characteristics have been mentioned only when of use for understanding the phenomena.

#### *Determination of Ammonia Nitrogen*

In solution: distillation with  $\text{MgO}$  of 50 ml. In soil: 50 g. of soil was shaken with 500 ml. of 1%  $\text{KCl}$  solution and aliquot parts distilled with  $\text{MgO}$ .

*Nitrite and Nitrate Nitrogen*

In the same extract the total amount of nitrite and nitrate N was determined according to the method of Arndt. In liquid cultures larger amounts of nitrite were determined iodometrically, and smaller quantities were determined colorimetrically according to the method of Griesz-Romyn.

*Total Nitrogen*

A sample of the well-mixed soil was ground in a ball mill until dust-fine to ensure homogeneity. This was necessary in order to get reproducible values and enabled us to make up accurate nitrogen balances at the different stages of the experiments. The total nitrogen was determined in 5 g. of soil by the method of Jodlbaur; as the greater part of the soils contained rather large amounts of nitrates, phenolsulphuric acid was added 12 hours before the proper determination.

*The pH*

The pH was determined in a watery suspension of 10 g. of soil in 50 ml. of water by means of a glass electrode; in a number of cases the buffer capacity was determined by thoroughly mixing the soil suspension with increasing quantities of  $\text{CaCO}_3$  and measuring the resulting pH's after 6 days.

**Results***Nitrogen Losses in Liquid Cultures of Nitrifying Bacteria*

After a few transfers in the sterile culture medium mentioned above, the pH of which had been raised to 6.5 by adding  $\text{Na}_2\text{CO}_3$  and which was aerated by means of a tube with a porous pumice stone, the results given in the first three rows of Table I were obtained. After ammonium sulphate solution was again added and the pH at 6.5 was re-established by adding  $\text{Na}_2\text{CO}_3$ , the values given in the last three rows of Table I were found.

TABLE I

Nitrification time (hours)	$\text{NH}_4^+\text{-N}$ in 100 ml.	$\text{NO}_2^- + \text{NO}_3^-\text{-N}$ in 100 ml.	Total inorganic N in 100 ml.	pH
0	21.4	85.6	107	6.5
24	16.0	91.8	107	6.3
72	9.2	97.8	107	6.3
0	30.4	92.4	121.8	6.5
24	23.4	98.4	121.8	6.5
48	16.2	106.6	121.8	6.5

It is evident that at a pH of 6.5-6.3 no nitrogen is lost, neither as  $\text{NH}_3$  nor as N-O compounds. After a fresh culture medium was inoculated with a few milliliters of the former culture and a good working culture was obtained, the pH was established at 6.0 by adding a quantity of  $\text{CaCO}_3$  amounting to about half the quantity eventually necessary to neutralize all the acid produced during nitrification of the ammonium sulphate present in the solution.

TABLE II  
NITROGEN LOSSES IN AN AERATED NITRIFICATION MEDIUM, pH BETWEEN 5 AND 6

Nitrification time (hours)	$\text{NH}_4^+\text{-N}^*$	$\text{NO}_2^- + \text{NO}_3^-\text{-N}^*$	Total inorganic $\text{N}^*$	N lost*	pH
0	39.0	74.8	113.8		6.0
24	29.2	74.6	104.8	9.0	5.2
0†	29.0	75.4	104.6		6.2
48†	13.6	75.4	88.8	15.8	5.2

\*Nitrogen is calculated as mg. in 100 ml. solution.

†The experiment was continued after the pH was restored to 6.2.

In the first stage of the experiment 90 mg. N/liter culture solution is lost and in the second part 158 mg. In total this amounts to 248 mg. N per liter within 3 days (see Table II). There is no reason to suppose that in this case nitrogen has been lost as ammonia, as this did not happen during the former experiments (Table I) at a higher pH of 6.5. One might also ask whether in the present case a certain amount of nitrogen has been used by the nitrifying bacteria to build up their bodies. However, nitrification is accomplished by a very small amount of bacterial material and because the circumstances of the last experiment are exactly the same (except for the pH) as in the former one, in which no nitrogen was lost, there is no reason to suppose that nitrogen has been withdrawn from the culture solution in this way. The only possible explanation is that in some unknown way nitrogen has been volatilized either as N—O compounds or as nitrogen. It is well known that at a pH of about 5, nitrites have an appreciable gaseous tension of NO, which rapidly oxidizes to  $\text{NO}_2$  when in contact with air. This nitrogen peroxide is oxidized by potassium permanganate both in alkaline or in acid solution to nitric acid. In order to determine whether during nitrification nitrogen escapes as NO, the air that had passed through the culture medium was scrubbed in a glass tube containing pumice stone drenched in alkaline potassium permanganate. At the end of the experiment the nitrogen content of the absorption material was determined. In the course of the investigation a modified Shaw scrubber (7) was used instead of the somewhat unhandy long tube with pumice. The results are shown in Table III.

In the first experiment 68 mg. N per liter were lost, 17.5 mg. of which was found in the permanganate solution. In this case the culture medium contained a rather large amount of nitrite at the beginning and it is not so surprising that during aeration when the pH dropped from 6.1 to 5.0, a certain amount of NO escaped from the solution. In the second experiment with a smaller nitrite content the total loss is smaller (24 mg./l.) as well as the amount of 6 mg. NO-N recovered with  $\text{KMnO}_4$ . In both cases however only about 25% of the total nitrogen lost is recovered as NO-N. So the question arises to what extent the alkaline permanganate solution absorbs the N—O compounds liberated from the nitrite solution. In order to answer this question

TABLE III

NITROGEN LOSSES IN AERATED CULTURE MEDIUM,  $\text{NO}_3$  COLLECTED IN  $\text{KMnO}_4$ 

Nitrification time (hours)	$\text{NH}_4^+\text{-N}$ , mg. in 100 ml.	$\text{NO}_2^-\text{-N}$ , mg. in 100 ml.	$\text{NO}_3^-\text{-N}$ , mg. in 100 ml.	Total inorganic N, mg. in 100 ml.	pH	Total $\text{NO}_3\text{-N}$ lost, mg./l.
0*	34.3	21.8	0.2	56.2	6.1	
24*	32.2	20.2	0.4	52.8	5.6	
48*	27.0	21.0	2.2	50.2	5.0	17.5
72*	26.0	20.0	2.8	49.4	4.9	
0†	40.4	10.0	33.6	84.0	6.7	
24†	36.4	12.0	35.2	83.6	6.3	
48†	30.6	6.0	45.8	82.4	5.5	
72†	24.0	0	58.0	82.0	5.1	6.0
96†	10.6	0	71.6	82.2	5.0	
120†	10.4	0	71.2	81.6	5.0	

\*Nitrification in this culture was rather slow, the pH dropped within 2 days from 6.1 to 5.0, at which pH nitrification nearly stopped.

†The experiment was repeated with a more vigorous culture which was better buffered.

equal portions of a 1% potassium citrate - HCl buffer solution were made up to different pH's, ranging from 3.0 to 6.0, 37 mg. N/100 ml. as  $\text{KNO}_3$  was added, the solution was aerated in the same way as the nitrification cultures, and the air scrubbed through  $\text{KMnO}_4$ . After 6 hours the residual nitrogen in the solution was determined as well as the N content of the permanganate. The results are shown in Table IV.

In these sterile buffer solutions containing inorganic nitrite increasing amounts of nitrogen are lost when the pH decreases (see Fig. 1). It is also clear that at pH's of 5.5-5.0, which occur in the nitrification cultures, appreciable amounts of nitrogen may be lost when nitrites are present, especially when one bears in mind that the nitrification experiments lasted much longer (up to 72 and 120 hours) than these aeration experiments (only 6 hours). In the case of the latter, 100% of the nitrogen lost is recovered in the permanganate scrubber, whereas in the nitrification experiments only 25% has been recovered. So it seems that in this case the greater part of the nitrogen lost has passed the permanganate solution unchanged and has escaped either as nitrogen gas or as nitrous oxide ( $\text{N}_2\text{O}$ ). As in the nitrification cultures, both ammonium sulphate and nitrite are present and there is the possibility of

TABLE IV

NITROGEN CONTENT OF NITRITE CONTAINING STERILE BUFFER SOLUTION AT DIFFERENT pH'S AND TOTAL N FOUND IN  $\text{KMnO}_4$  SOLUTION AFTER 6 HOURS' AERATION

pH	Nitrogen content (mg./100 ml.)		Recovered N in $\text{KMnO}_4$	% lost
	Before	After		
3.0	37.0	27.4	9.6	26
3.5	37.0	31.5	5.5	15
4.0	37.0	33.7	3.3	9
4.5	37.0	35.2	1.8	5
5.0	37.0	36.1	0.9	2.5
5.5	37.0	36.7	0.3	1
6.0	37.0	37.0	0	0

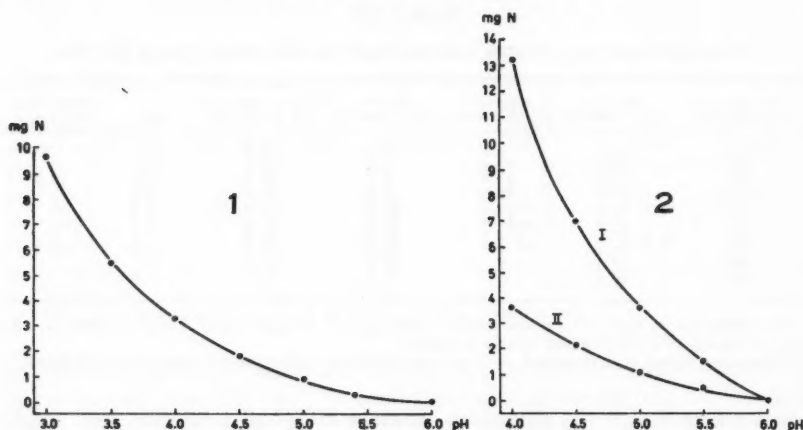


FIG. 1. Nitrogen losses during 6 hours' aeration of KNO<sub>2</sub> solutions of different pH's. All nitrogen volatilized could be recovered in alkaline permanganate and consisted of NO.

FIG. 2. Nitrogen lost during 6 hours' aeration of a solution containing a mixture of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and KNO<sub>2</sub> at different pH's. (Curve I, total losses.) Only about 30% of the total N lost could be recovered in alkaline permanganate (Curve II), the rest consisted of nitrogen gas.

such losses by chemical reaction. In order to verify the influence of the pH on such losses, sterile buffer media of different pH's, containing small amounts of ammonium sulphate and potassium nitrite, were aerated; the air that had passed through the solution was washed in a permanganate scrubber. The buffer solution contained 30–35 mg./100 ml. KNO<sub>2</sub>-N and 40–45 mg./100 ml. (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>-N; the pH's were varied from 4.0 to 6.0 and the aeration was continued during 6 hours. The results are summarized in Table V.

TABLE V  
NITROGEN LOSSES AT DIFFERENT pH'S IN STERILE BUFFER SOLUTIONS CONTAINING  
NITRITE AND AMMONIUM SULPHATE, AFTER 6 HOURS' AERATION

pH	NH <sub>4</sub> -N, mg./100 ml.	NO <sub>2</sub> -N mg./100 ml.	N recovered in permanganate, mg./100 ml.	NH <sub>4</sub> -N lost, mg./100 ml.	NO <sub>2</sub> -N lost, mg./100 ml.	Total N lost, mg./100 ml.	N lost as N <sub>2</sub> in % total loss
4.0	44.8*	35.8*	—	—	—	—	—
4.0	40.0	27.4	3.6	4.8	8.4	13.2	73
4.5	42.3	31.3	2.1	2.5	4.5	7.0	70
5.0	43.6	33.4	1.2	1.2	2.4	3.6	67
5.5	44.3	34.3	0.5	0.5	1.0	1.5	67
6.0	44.8	35.8	0	0	0	0	0

\*Original solution.

This experiment proves that when a sterile diluted solution of potassium nitrite and ammonium sulphate is aerated at different pH's, only a small part of the nitrogen lost is absorbed by permanganate and consists of NO, whereas the greater part is lost as nitrogen gas or perhaps to some extent as N<sub>2</sub>O (see Fig. 2). The gas produced in a similar experiment was analyzed and proved to consist of 56% and 44% N<sub>2</sub>; N<sub>2</sub>O however was absent.



Throughout the experiment the percentage of  $N_2$  calculated on the total inorganic N lost is nearly constant, amounting to about 70% irrespective of the pH, only 30% being accountable as NO. These losses decrease when the pH increases and stop entirely at a pH of 6 and above. This removes all doubt that ammonia as such could be one of the constituents of the gas, as in that case the amount would increase at higher pH's. It is to be expected that in this respect the sterile solution behaves in the same way as the nitrification cultures. It seems logical to conclude that in the latter case also the losses are due to the simultaneous presence of ammonium and  $HNO_2$  in the culture solution, which finally react in a purely chemical way to produce nitrogen gas, whereas a smaller amount escapes as NO, which during aeration is oxidized to  $NO_2$ . Finally a nitrogen balance was made of ordinary nitrification cultures performed as usual in shallow layers in Erlenmeyer flasks, without extra aeration. The pH of one series was well buffered between 7.0 and 6.5; of the other series the buffer capacity was adjusted in such a way that a drop of the pH from 6 to 5 could be expected as a result of the acids produced during nitrification. The results are summarized in Table VI.

TABLE VI  
NITROGEN BALANCE OF NITRIFICATION IN SHALLOW LAYERS IN ERLENMEYER  
FLASKS (MG. N/LITER)

Experiment	pH	$NH_4^+-N$	$NO_2^--N$	$NO_3^--N$	Total mineral N
I					
At the beginning	7.0	628	7.0	33	668
After 1 week	6.6	502	57	109	668
Difference		-126	+50	+76	0
II					
At the beginning	6.0	424	6.0	46	476
After 1 week	5.3	352	2.0	96	450
Difference		-72	-4.0	+50	-26

In the well-buffered culture solution no losses occurred. In the other however, an appreciable amount of nitrogen was lost. It is to be noted that the quantity of nitrogen lost (26 mg.) is far in excess of the quantity of nitrite N lost, which means that these losses originate from nitrite that has been produced during the experiment. It is well known that although in alkaline media there is an accumulation of nitrite in the beginning (see Experiment I), the nitrite produced in acid media by the nitrite bacteria is so rapidly oxidized by the nitrate bacteria that it can hardly be detected. Perhaps it is better to say that the ratio of the velocity of the nitrataion to that of the nitrification changes under influence of the pH and decreases when the pH increases, whereas the absolute values of both decrease as the pH becomes more acid. Notwithstanding the fact that in acid culture media hardly any nitrite can be detected, the chemical reaction between these traces of  $HNO_2$  and ammonia proceeds to a measurable extent, giving rise to considerable

nitrogen losses. It is remarkable that, although for many decades nitrification experiments have been carried out in shallow layers, nitrogen losses have never been observed, most probably because in general either  $\text{MgCO}_3$  or  $\text{CaCO}_3$  have been added to keep the pH at optimal values.

#### *Nitrogen Balances of Acid Sandy Soils*

In order to be better informed on the conditions under which nitrogen losses occur in soils, several soils were stored in Mitscherlich pots at 24° C. after the soil was dressed with ammonium sulphate (40 mg. N/100 g. of dry soil). From previous experiments it seemed plausible that the initial pH and the buffer capacity of the soil were of great importance for the occurrence of nitrogen losses. Consequently in addition to the experiment with the original soil some experiments were carried out with the same soil to which varying amounts of  $\text{CaCO}_3$  had been added. In the following experiment, which has to be regarded as a first orientation, four Mitscherlich pots were filled with 5 kg. of an acid sandy soil each. The humus content of the soil was 6.4%, its original pH 5.9, and the C/N ratio of the organic matter in the soil 20. Two of the pots received 25 g.  $\text{CaCO}_3$ , which raised the pH to 7.1.

In this case, in the original soil without any addition except the ammonium sulphate, 18.5% of the ammonium N added is lost within 4 weeks, which increases to 28% after 8 weeks. The addition of  $\text{CaCO}_3$  stops the nitrogen

TABLE VII

Nitrification time (weeks)	NH <sub>4</sub> <sup>+</sup> -N, mg./100 g. soil	NO <sub>3</sub> <sup>-</sup> -N	Organic N	Total N	Lost, N			pH
					Mg.	% of the added NH <sub>4</sub> <sup>+</sup> -N	Organic N mineralized	
Original soil without CaCO <sub>3</sub>								
0	43.7	8.5	141.1	191	0	0	—	5.9
	42.6	6.9		193				
	43.2	7.7		192				
4	24.8	28	129.6	183	8	18.5	11.5	4.5
	26.0	30		185				
	25.4	29		184				
8	23.7	31	124.1	179	12	28	17.0	4.4
	24.0	33		180				
	23.9	32		180				
25 g. CaCO <sub>3</sub> per pot								
0	39.5	7.9	140.9	188	—	—	—	7.1
	37.3	9.4		187				
	38.4	8.7		188				
4	0.5	47	139.3	187	0	0	1.6	6.1
	0.9	48		188				
	0.7	48		188				
8	0	55	134.0	187	0	0	6.9	5.9
	0	53		188				
	0	54		188				

losses entirely and it seems that in the pH range between 7.1 and 5.9, circumstances are unfavorable for volatilization. In this case all the ammonium sulphate added is nitrified and even 6.9 mg. more. The latter have originated from the organic matter in the soil. In the former case, 19.3 mg.  $\text{NH}_4^+\text{-N}$  has disappeared and 24.3 mg.  $\text{NO}_3^-\text{-N}$  are formed after the experiment. When we assume that half of the nitrogen which has been lost originates from nitrite, the total quantity of ammonia nitrogen that has been nitrified amounts to  $24.3 + 12/2 = 30.3$  mg. This is  $30.3 - 19.3 = 11$  mg. more than the ammonia N which has disappeared, so in this case also part of the nitrogen lost came from the soil organic matter. In Table VII the duplicate determinations have been listed in order to give some idea of the experimental errors; it is evident that the mean error of the total nitrogen determination is small and that the losses of 8 and 12 mg. N are far in excess of the experimental error. As the pH seems to play an important role in these processes it was necessary to adjust the quantity of  $\text{CaCO}_3$  to be added in accordance with the buffer capacity of the soil and the quantity of acid to be expected from the nitrification of the ammonium sulphate. To this end different portions of 100 g. of soil, to which the nutrients had been added, were mixed with increasing quantities of  $\text{CaCO}_3$  and, after having been thoroughly mixed, left 1 week to react, after which the pH's were determined. The resulting pH's were plotted against the quantities of  $\text{CaCO}_3$  and from these curves the quantities of  $\text{CaCO}_3$  necessary to obtain suitable pH values could be read. That these quantities can diverge widely is shown in the following case where two different soils have been treated in this way. To raise the pH of soil I from 4 to 6, 1.8 g.  $\text{CaCO}_3$  was necessary, whereas in the case of soil II 3.2 times as much (5.8 g.) was required. In the following tables the quantities of organic nitrogen are listed too and these have been calculated by subtracting the sum of the inorganic nitrogen quantities from the total nitrogen.

TABLE VIII

NITROGEN BALANCE OF A SOIL TO WHICH INCREASING QUANTITIES OF  $\text{CaCO}_3$  HAVE BEEN ADDED (N IN MG./100 G. OF DRY SOIL)

(Sandy soil, humus content 4.4%, C/N ratio of soil organic matter 23.7, initial pH 5.3)

Time, days	$\text{CaCO}_3$ , g./kg.	$\text{NH}_4^+\text{-N}$	$\text{NO}_2^-\text{-N}$	$\text{NO}_3^-\text{-N}$	Organic N	Total N	N lost		pH
							Mg./ 100 g.	% of added $\text{NH}_4^+\text{-N}$	
0	0	36.0	0.3	3.9	107.8	148	—	—	5.3
26	0	36.0	0	2.3	109.7	148	—	—	5.2
49	0	37.2	0.3	4.8	106.7	149	—	—	4.7
77	0	36.5	0	5.9	105.6	148	—	—	4.6
0	3.2	36.0	0.8	2.2	105.0	144	—	—	6.4
26	3.2	28.0	0	3.4	112.6	144	—	—	5.9
49	3.2	17.9	0.3	17.9	106.9	143	—	—	5.5
77	3.2	5.8	0	22.0	102.2	130	14	39	4.4
0	5.3	35.2	1.2	2.3	105.3	144	—	—	6.6
26	5.3	24.4	4.9	0.9	111.8	142	—	—	6.8
49	5.3	1.1	0.3	13.4	128.3	143	—	—	5.9
77	5.3	1.1	0	40.9	100.0	142	—	—	6.0

In the original soil, without the addition of  $\text{CaCO}_3$ , nitrification is entirely at a standstill and consequently there are no nitrogen losses. Raising the pH to 6.4 with a moderate quantity of  $\text{CaCO}_3$  improves the conditions considerably. After 77 days nearly all the ammonia has been nitrified. In the last period (49th to 77th day) during which the pH dropped from 5.5 to 4.4, 39% of the ammonia N added in the beginning is lost. When we calculate it on the ammonia N present after 49 days, i.e. at the beginning of the last period, 78% has been volatilized. The fact that during this last period 14 mg. N has been lost and only 0.3 mg.  $\text{NO}_2^-$ -N disappeared is important as it proves that an accumulation of nitrite is no *conditio sine qua non* for volatilization of nitrogen. One gets the impression that even when the nitrite stage of nitrification is hardly detectable, the chemical reaction which causes the nitrogen losses monopolizes the greater part of the nitrite as soon as it is formed and in this way prevents nitrification to its natural final stage, nitratation. In the case of the highest dose of  $\text{CaCO}_3$  all the ammonia added disappeared within 49 days. At that time only  $13.4 + 0.3 - (2.3 + 1.2) = 10.2$  mg. N or 29% is found as nitrite-nitrate N, whereas 68% has been incorporated in microbial cell material. This incorporation caused the organic nitrogen to increase from 105.3 to 128.3 mg. per 100 g. soil. In the last period not less than 27% of the organic nitrogen has been ammonified and subsequently nitrified. From this experiment one gets the impression that the pH zone which favors the volatilization lies between 5.5 and 4.3. In the next experiment (Table IX) the same tendency is observed in a sandy soil (91.7% sand) with only 3.9% humus and an initial pH of 5.0. Owing to the low pH, nitrification in the original soil proceeds only very slowly and except for a few milligrams no nitrogen is lost.

TABLE IX

NITROGEN BALANCE OF A SOIL IN WHICH HIGH NITROGEN LOSSES ARE INDUCED BY THE ADDITION OF A SMALL QUANTITY OF  $\text{CaCO}_3$

Time, days	$\text{CaCO}_3$ , g./kg.	$\text{NH}_4^+$ -N, mg./100 g.	$\text{NO}_2^- + \text{NO}_3^-$ -N, mg./100 g.	Organic N, mg./100 g.	Total N, mg./100 g.	N lost		pH
						Mg./100 g.	% $\text{NH}_4^+$ -N added	
0	0	30.3	1.0	134.7	166	—	—	5.0
21	0	30.3	1.5	134.2	166	—	—	4.8
42	0	27.2	4.2	134.6	166	—	—	4.8
58	0	24.0	6.3	134.7	165	—	—	4.6
77	0	24.8	7.6	132.6	165	—	—	4.4
99	0	25.2	8.8	130.0	164	2	6	4.3
0	2.5	30.0	1.0	135.0	166	—	—	6.7
21	2.5	28.3	3.8	134.9	166	—	—	6.0
42	2.5	21.7	9.2	135.1	166	—	—	6.0
58	2.5	17.3	15.9	132.8	166	—	—	5.9
77	2.5	12.0	20.2	114.8	147	19	64	5.3
99	2.5	9.6	23.1	113.3	146	20	67	5.0
0	5	30.5	1.0	134.5	166	—	—	6.9
21	5	22.8	4.8	138.4	166	—	—	6.5
42	5	10.7	15.6	139.7	166	—	—	6.5
58	5	5.3	24.4	136.3	166	—	—	6.4
77	5	0	27.4	138.6	166	—	—	6.2
99	5	0.5	27.3	137.2	165	—	—	5.9

By adding 2.5 g.  $\text{CaCO}_3$  per kg. of soil the bacteriological processes in this soil are markedly stimulated. After 77 days 64% of the ammonia nitrogen added is nitrified and 16% of the organic nitrogen originally present is ammonified. Assuming that half of the nitrogen lost originates from the nitrite, all the ammonia nitrogen added and part of the ammonia derived from the soil organic matter has been nitrified. By addition of 5 g.  $\text{CaCO}_3$  per kg. of soil, the pH increases to 6.9 and remains high until the end of the experiment. This prevents the formation of  $\text{HNO}_2$  and consequently volatilization as well. That ammonification is also prevented is somewhat surprising, as it is generally accepted that it is accelerated after the addition of  $\text{CaCO}_3$  to a humus-containing soil. As the soil in its original state was acid, the main population will have consisted of fungi; raising the pH to 6.9 makes the conditions for most fungi less favorable. In the experiment with 2.5 mg.  $\text{CaCO}_3$  ammonification starts at a pH between 5.9 and 5.3, a region which is not reached in the latter case. One gets the impression that in such acid humus-containing soils also ammonification is limited to certain pH regions. In this case, as well as in the former one, only one factor is responsible for the appearance of the losses, i.e., the addition of a small quantity of  $\text{CaCO}_3$ . The principal effect caused by the  $\text{CaCO}_3$  is an increase of the pH to a value (6.7) that is favorable for a rapid nitrification and such a change of the buffer capacity of the soil that during nitrification the pH is gradually lowered to 5.0. Between 5.9 and 5.3 not less than 64% of the nitrogen added as ammonium sulphate has been lost. In this case also nitrogen losses coincide with a strong ammonification, 14% of the total organic nitrogen being ammonified in that period. It is questionable whether both phenomena are interrelated or are independent of each other, the increased activity of nitrifying bacteria and ammonifying microorganisms being due to a favorable pH. In the previous cases the original soils used for the experiments did not show any nitrogen losses unless small amounts of  $\text{CaCO}_3$  were added. Therefore, although the experiments until now described enlarged our insight considerably, the question remains whether there are any soils which show these losses normally without the addition of  $\text{CaCO}_3$ . The following cases show that there are indeed such soils. The first case (Table X) was a sandy soil, 81.2% sand, 5.3% clay, pH 4.0, humus content 13.1%, 400 mg. organic nitrogen/100 g., and a C/N ratio in the organic matter of 18.2.

Although the pH of this soil is as low as 4.0, the nitrifying bacteria seem to be adapted to this situation; after 3 months 6.7 mg.  $\text{NO}_2^- + \text{NO}_3^-$ -N had been formed (10.7-4.0), and as at least half of the nitrogen lost must have passed through the nitrite stage, 7½ mg. more would have to be included making a total amount of 14.2 mg. of N nitrified or 52% of the ammonia N added. The addition of a moderate quantity of  $\text{CaCO}_3$  accelerates not only nitrification, but ammonification and volatilization as well, the latter being more than doubled. When 7 g. of  $\text{CaCO}_3$  per kg. of soil is added, nitrification and ammonification are quickened too, but volatilization is retarded. This is because the pH had risen to 5.9 and the buffer capacity had increased so

TABLE X

NITROGEN BALANCE OF AN ACID SOIL WHICH SHOWS CONSIDERABLE NITROGEN LOSSES IN ITS UNLIMED STATE

Time, days	CaCO <sub>3</sub> , g./kg.	NH <sub>4</sub> <sup>+</sup> -N, mg./100 g.	NO <sub>2</sub> <sup>-</sup> + NO <sub>3</sub> <sup>-</sup> -N, mg./100 g.	Organic N, mg./100 g.	Total N, mg./100 g.	N lost		Organic N mineralized, mg./100 g.	pH
						Mg./100 g.	% of added NH <sub>4</sub> <sup>+</sup> -N		
0	0	27.4	4.0	409.6	441	—	—	—	4.0
19	0	33.0	6.9	401.1	441	—	—	8.5	4.0
38	0	26.8	6.8	407.4	441	—	—	2.2	4.0
58	0	22.2	5.2	401.6	439	2	7	8.0	4.0
70	0	20.1	6.2	400.7	427	14	51	8.9	4.0
91	0	26.7	10.7	388.6	426	15	55	21.0	4.0
0	4	27.0	8.0	407.0	442	—	—	—	5.4
19	4	27.9	8.6	404.5	441	—	—	2.5	5.3
38	4	22.3	11.9	403.8	438	4	11	3.2	5.1
58	4	11.2	17.1	392.7	421	21	78	14.3	4.8
70	4	5.6	33.5	368.9	409	33	112	38.1	4.5
91	4	2.1	30.7	376.2	409	33	112	30.8	4.4
0	7	27.8	5.3	407.9	441	—	—	—	5.9
19	7	35.7	11.5	393.8	440	—	—	14.1	5.7
38	7	33.5	9.8	396.7	440	—	—	11.2	5.8
58	7	17.0	28.7	394.3	440	—	—	13.6	5.2
70	7	8.3	29.0	382.7	420	21	78	25.2	4.8
91	7	2.5	34.2	373.3	410	31	115	34.6	4.6

much that it took more time to bring the pH down to the region where volatilization becomes possible. In both cases it sets in at a pH below 5.2. As more nitrogen disappeared than had been added as ammonium sulphate, part of the nitrogen lost originates from the ammonia produced by ammonification of the soil organic matter. In this case also ammonification is quite vigorous and one is confronted again with the question of whether both processes are linked to each other or are quite independent. In the case of the soil without CaCO<sub>3</sub>, in the period between 58–70 days, 46% of the total ammonia has been volatilized, whilst only 0.9 mg. N had been ammonified; in the period between the 70th and 91st day 12.1 mg. N are ammonified and only 1 mg. N is volatilized. In the experiment where 7 g. of CaCO<sub>3</sub> had been added, after 58 days of incubation 13.6 mg. of N had been ammonified, but not a single milligram of N was volatilized, although the pH was 5.2 at that time and most of the ammonia added had been nitrified. So it seems possible that volatilization and ammonification proceed quite independently of each other which does not exclude the possibility that both processes affect each other in an indirect way. In this connection it is of interest to reconstruct the path along which the different forms of nitrogen reached their destiny in the case where 4 g. of CaCO<sub>3</sub> was applied. Between the 38th and the 58th day, 11.1 mg. NH<sub>4</sub>-N disappeared, 5.2 mg. of which were nitrified, 5.9 mg. being left for volatilization. However 17 mg. of N were lost, which means that 17 – 5.9 = 11.1 mg. originated from the organic nitrogen ammonified. All the organic nitrogen ammonified in this period has been volatilized. In the next period (58th–70th day) 5.6 mg. NH<sub>4</sub>-N disappeared and as 16.4 mg. of N was nitrified, 10.8 mg. of this amount must have been derived from the soil organic matter. Moreover 12 mg. of N were volatilized and these also originated from the organic nitrogen. This is an



important fact because in agricultural practice acid sandy soils like these are generally not dressed with ammonium sulphate, and although there is no external source of ammonia, the nitrogen of the soil organic matter itself after having been ammonified and partly nitrified, can fall a victim to volatilization. For better understanding of the circumstances which favor volatilization it might be of use to analyze also a case where no losses were observed, although at first sight conditions necessary for such losses were apparently fulfilled (Table XI). The soil in question showed in outline the same characteristics as the former one of Table X: sandy soil, 82.5% sand, 7.2% clay, pH 4.4, humus content 10.3%, 335 mg. N/100 g. of soil in the organic matter, and a C/N ratio of 14.4.

TABLE XI

NITROGEN BALANCE OF A SOIL WHICH DID NOT SHOW ANY NITROGEN LOSSES  
(MG. N/100 G. OF SOIL)

Time, days	CaCO <sub>3</sub> , g./kg.	NH <sub>4</sub> <sup>+</sup> -N	NO <sub>3</sub> <sup>-</sup> +NO <sub>2</sub> <sup>-</sup> -N	Organic N	Total N	N lost		Organic N mineralized*	pH
						Mg./100 g.	% of added NH <sub>4</sub> <sup>+</sup> -N		
0	0	28.4	2.0	334.6	365	—	—	—	4.4
19	0	31.8	3.6	329.6	365	—	—	5.0	4.2
38	0	31.5	6.7	325.8	364	—	—	9.8	4.2
58	0	32.5	7.0	325.5	365	—	—	9.1	4.2
79	0	30.6	11.9	323.0	366	—	—	11.6	4.2
100	0	29.9	8.0	329.1	367	—	—	5.5	4.3
0	2.5	28.6	2.4	334.0	365	—	—	—	5.3
19	2.5	31.2	4.8	330.0	366	—	—	4.0	5.5
38	2.5	27.6	5.6	333.8	367	—	—	0	5.0
58	2.5	23.1	7.5	335.4	366	—	—	-1.4	4.9
79	2.5	21.6	12.8	331.6	366	—	—	2.4	4.7
100	2.5	18.6	13.0	333.4	365	—	—	0.6	4.7
0	5	30.0	3.1	333.9	367	—	—	—	6.0
19	5	38.2	3.2	323.6	365	—	—	10.3	5.9
38	5	37.0	10.5	317.5	365	—	—	16.4	5.8
58	5	30.4	11.7	324.4	366	—	—	9.5	5.5
79	5	23.4	19.0	321.7	365	—	—	12.2	5.1
100	5	17.6	22.1	327.3	366	—	—	6.6	4.8

\*A negative figure under the heading "Organic N mineralized" means that instead of mineralization nitrogen has been assimilated.

Although the circumstances in this soil, especially the pH and the buffer capacity, are favorable for volatilization, no nitrogen losses were observed. On closer inspection of the available data there are, however, some important differences. In the first place nitrification in this soil is less vigorous than in the former ones. This is most pronounced in the experiment which received 2.5 g. of CaCO<sub>3</sub> per kg., for in 79 days only 10.4 mg. of nitrate N was formed, whereas in the corresponding case of Table X 25.4 mg. was produced in 70 days. Adding hereto half of the nitrogen lost, it would make a total of 41.9 mg., i.e. four times as much. Also, ammonification of the organic nitrogen is very slow and not more than half or one third of that of the former experiment. As the C/N ratio in the present experiment is 14.4, it is to be expected that the soil organic matter is in a more stable state than that of the former experiment, the C/N ratio there being 18.2. Consequently the general impression of the present soil is that its microbial activity is much

lower than that of the soils where considerable nitrogen losses were observed. Although a strong ammonification does not seem to be an indispensable factor for volatilization, one gets the impression that the largest losses occur in soils where both nitrification and ammonification proceed vigorously. The coincidence of ammonification, nitrification, and volatilization is quite apparent in the following cases, which in both their natural state and after the addition of  $\text{CaCO}_3$  lose considerable quantities of nitrogen. The characteristics of the soil used in the first of these experiments were: sand 88.2%, clay 7.6%, humus 4.8%, pH 4.5, organic nitrogen 155.5 mg./100 g. of soil, C/N ratio of the organic matter 18.6. The data of this experiment are recorded in Table XII.

TABLE XII  
NITROGEN BALANCE OF A SOIL WHICH SHOWS FAIR NITROGEN LOSSES COINCIDING  
WITH STRONG AMMONIFICATION (MG. N/100 G. OF SOIL)

Time, days	$\text{CaCO}_3$ g./kg.	$\text{NH}_4^{+}\text{-N}$	$\text{NO}_2^{-} + \text{NO}_3^{-}\text{-N}$	Organic N	Total N	N lost			pH
						Mg./100 g.	% of $\text{NH}_4^{+}\text{-N}$ added	Organic N mineralized	
0	0	29.9	2.6	155.5	188	—	—	—	4.5
19	0	37.6	2.8	147.6	188	—	—	7.9	4.5
38	0	34.2	1.9	152.9	189	—	—	2.6	4.4
58	0	31.4	2.3	153.3	187	—	—	2.2	4.4
79	0	32.9	7.0	138.1	178	10	34	17.4	4.4
100	0	36.2	4.8	136.0	177	11	37	19.5	4.5
0	2.5	27.1	2.9	157.0	187	—	—	—	6.5
19	2.5	33.9	6.3	148.8	189	—	—	8.2	6.3
38	2.5	30.4	5.8	151.8	188	—	—	5.2	6.1
58	2.5	23.2	8.3	156.5	189	—	—	0.5	5.8
79	2.5	20.8	17.0	139.2	177	10	37	17.8	5.6
100	2.5	17.2	16.5	142.3	176	11	41	14.7	4.9
0	5	27.8	1.7	157.5	187	—	—	—	6.9
19	5	27.8	3.2	158.0	188	—	—	—	6.9
38	5	25.3	2.8	159.9	188	—	—	—	6.8
58	5	13.2	21.4	153.4	188	—	—	4.1	6.2
79	5	5.6	30.0	141.4	177	10	36	16.1	5.6
100	5	2.2	32.5	142.3	177	10	36	15.2	5.4

Irrespective of the addition of  $\text{CaCO}_3$ , volatilization coincides with ammonification and the question is raised whether intermediary N compounds produced during ammonification are more suited for volatilization than the ammonia from ammonium sulphate. It is also to be noted that the pH regions in which nitrogen has been lost were respectively 4.4–4.4, 5.8–5.6, and 6.2–5.6, and in the latter case especially the nitrogen loss is higher than in all the previous experiments. One might wonder why, in the last stage (79th–100th day) in the soil receiving 5 g. of  $\text{CaCO}_3$ , no nitrogen has been volatilized even though the pH was favorable. The reason is that practically all ammonia had disappeared after 79 days and consequently no nitrite was being formed. In Table XIII the data are recorded of an experiment which demonstrated the largest nitrogen losses observed during this investigation. Characteristics of this soil are: sand 89%, clay 6.2%, pH 4.4, humus content 4%, C/N ratio of soil organic matter 15.6, organic nitrogen 127.5 mg./100 g. of soil.

TABLE XIII

NITROGEN BALANCE OF A SOIL WHICH SHOWED MAXIMUM NITROGEN LOSSES COINCIDING WITH INTENSIVE MINERALIZATION (MG. N/100 G. OF SOIL)

Time, days	CaCO <sub>3</sub> , g./kg.	NH <sub>4</sub> <sup>+</sup> -N	NO <sub>2</sub> <sup>-</sup> +NO <sub>3</sub> <sup>-</sup> -N	Organic N	Total N	Total N lost			pH
						Mg.	% NH <sub>4</sub> <sup>+</sup> -N added	Organic N mineralized	
0	0	35.0	0.8	128.2	164	—	—	—	4.4
21	0	33.8	1.9	128.6	164	—	—	—	4.5
48	0	29.7	1.7	132.6	164	—	—	-4.4*	4.4
59	0	19.9	5.1	140.0	165	—	—	-11.8	4.4
78	0	23.2	4.5	110.3	138	26	74	17.9	4.4
100	0	18.5	5.1	113.4	137	27	77	14.8	4.4
0	2.5	35.5	1.2	127.3	164	—	—	—	6.3
21	2.5	33.8	3.7	126.5	164	—	—	0.8	6.1
48	2.5	27.5	7.2	129.3	164	—	—	-2.0	5.5
59	2.5	19.8	16.3	128.9	164	—	—	-1.6	5.1
78	2.5	16.0	23.2	100.8	140	24	68	26.5	5.1
100	2.5	12.3	21.3	106.4	140	24	68	10.9	4.7
0	5	37.0	0.9	127.1	165	—	—	—	6.8
21	5	30.2	8.7	125.1	164	—	—	2.0	6.8
48	5	19.0	12.7	133.3	165	—	—	-5.2	6.4
59	5	7.6	24.7	131.7	164	—	—	-4.6	5.8
78	5	2.2	34.0	116.8	153	12	33	10.3	5.4
100	5	2.3	35.1	105.6	143	22	60	21.5	5.2

\*Negative values indicate that nitrogen has been assimilated instead of mineralized.

The most striking fact of this experiment is that the soil in its original state loses 77% of the nitrogen added as ammonium sulphate, which coincides with the mineralization of about 10% of the organic nitrogen present. Adding 2.5 g. of CaCO<sub>3</sub>/kg. of soil intensifies mineralization whereas volatilization is somewhat slowed down. In the last case with 5 g. of CaCO<sub>3</sub>/kg. of soil both volatilization and ammonification are smaller than in the previous cases, whereas nitrification has been much quicker. In the period between the 78th and 100th day the 10 mg. of N volatilized originated from the organic nitrogen, which after mineralization must have been nitrified. In all the experiments a certain amount of nitrogen has been assimilated initially by the microorganisms. In the soil without CaCO<sub>3</sub> this amounted to 11.8 mg. after 59 days; 3 weeks later this quantity plus 17.9 = 29.7 in total has been mineralized. The same happened in the soil with 2.5 g. of CaCO<sub>3</sub> and also in the last series, although in a lesser degree. One gets the impression that in this soil microbial life is quite intense and the organic matter is not yet stabilized, although the C/N ratio of 14.4 is rather narrow. It was observed that in the period from the 78th to the 100th day in the third part of the experiment the amount of ammonia and that of the nitrite + nitrate-N did not change notably, whereas the amount of organic N mineralized increased from 10.3 to 21.5 mg. This observation gave rise to the question of whether the nitrogen lost originated directly from the organic nitrogen, without the action of the nitrite bacteria. However, since this was not very probable, it might also be assumed that part of the ammonia produced by ammonification must have been nitrified and oxidized to HNO<sub>2</sub>, the latter reacting with the other part of the ammonia liberated from the organic matter. Finally a series of pot experiments was made, part of which were sown to

*Brassica napus*. In this case extreme caution had been taken to homogenize the soil. The soil samples taken from these pots were ground dust-fine in a ball mill to ensure homogeneity. In order to be able to make accurate nitrogen balances the nitrogen assimilated by the plants was determined too, and calculated as milligrams of N assimilated from 100 g. of dry soil. The experiments were carried out in Mitscherlich pots containing 5 kg. of soil, each dressed with 1 g. of  $\text{KH}_2\text{PO}_4$ , 0.1 g. of  $\text{Na}_2\text{HPO}_4$ , 0.3 g. of  $\text{K}_2\text{SO}_4$ , 0.3 g. of  $\text{MgSO}_4$ . Nitrogen was added as  $(\text{NH}_4)_2\text{SO}_4$  or  $\text{KNO}_3$  in quantities denoted in the tables (30–40 mg. of N per 100 g. of dry soil). In the pots sown to *Brassica napus*, after germination, four of the best plants were selected. In some of the pots the soil had been baked for 1 hour at  $75^\circ \text{C}$ ., in order to eliminate the nitrifying bacteria. The pots were regularly weighed and the water content kept at about 60% of the water capacity. Since in this rather acid soil a weak activity of microbes could be presumed, 2% of a fertile garden soil has been added to part of the pots. Finally the following scheme of the experiment was adopted:

- 1A. The original soil dressed as indicated above, nitrogen applied as ammonium sulphate.
- 1B. The same as 1A, but pasteurized.
- 1C. The same as 1A, but planted with *Brassica napus*.
- 2A. The same as 1A, but infected with 2% of garden soil.
- 2B. The same as 2A, 15.9 g. of  $\text{CaCO}_3$  added.
- 2C. The same as 2A, planted with *Brassica napus*.
3. The original soil dressed as indicated above, nitrogen applied as sodium nitrate.

Characteristics of the soil are: 95.6% sand, 4.4% clay, 7.3% humus, 98.9 mg. of N/100 g. in organic substances, pH 4.8.

It is evident that addition of fertile garden soil has intensified microbiological activity notably; not less than 78% of the ammonia nitrogen present in the beginning has been volatilized within 80 days and also ammonification has been somewhat quickened. That after 25 days 10.6 mg. of N has been volatilized and only 1.9 mg. of N had been ammonified indicates once more that volatilization need not be coupled with ammonification. Adding  $\text{CaCO}_3$  intensifies nitrification but entirely stops volatilization, in the first place because the pH is raised above the critical margin. In the last period however, when the pH drops from 5.7 to 5.2, one might expect some nitrogen losses, more so as in that period an appreciable amount of ammonia N has been nitrified (10.1 mg.). Owing to the change in pH, the composition of the microbial flora changes, promoting the bacteria above the fungi, which is reflected by a rather strong assimilation of nitrogen. When a crop is grown on the soil, volatilization is reduced as we have also observed in Expt. 1C of Table XIV. In this case, however, there is no doubt at all that the diminution of volatilization is caused by the monopolization of the inorganic nitrogen by the plant. In the period between the 50th and 73rd day the

TABLE XIV

NITROGEN BALANCE IN THE ORIGINAL SOIL AND IN THE SAME SOIL CULTIVATED OR PASTEURIZED (MG. OF N IN 100 G. OF SOIL (DRY)) (EXPTS. 1A, 1B, AND 1C)

Time, days	NH <sub>4</sub> <sup>+</sup> -N	NO <sub>3</sub> <sup>-</sup> +NO <sub>2</sub> <sup>-</sup> -N	Organic N	Total N†	Total N lost		Organic N mineral- ized‡	N in plants*	pH
					Mg.	% NH <sub>4</sub> <sup>+</sup> -N added			
Expt. 1A—soil + ammonium sulphate									
0	45.6	2.4	98.9	146.9	—	—	—	—	4.8
30	40.3	6.7	97.4	144.4	2.5	5.5	1.5	—	4.7
50	30.0	6.7	95.6	132.3	14.6	32	3.3	—	4.5
73	28.9	9.0	88.0	125.9	21.0	46	10.9	—	4.2
87	30.8	9.8	85.8	126.4	20.5	45	13.1	—	4.1
Expt. 1 B—soil pasteurized§									
0	45.6	2.4	98.9	146.9	—	—	—	—	4.8
30	42.1	1.7	103.0	146.8	0	—	-4.1	—	5.0
50	37.7	0.8	108.8	147.3	0	—	-10.1	—	5.0
73	46.3	1.2	98.0	145.5	0	—	0.9	—	4.8
Expt. 1C—soil + ammonium sulphate, planted									
0	45.6	2.4	98.9	146.9	—	—	—	—	4.8
30	36.5	6.0	101.5	144.6	2.3	5	-2.6	0.6	4.7
50	21.3	3.6	101.8	133.2	13.7	30	-2.1	6.5	4.5
73	4.8	0.2	104.5	129.3	17.6	39	-5.6	19.5	3.9

\*Calculated for 100 g. of dry soil.

†In the total N, the N of the plants has been included.

‡Negative values indicate that N has been assimilated by microorganisms.

§Pasteurizing the soil entirely stops volatilization, indicating that it depends upon microbial processes. Although the nitrifying bacteria have been killed, other microorganisms may have withstood 75° C. and these are responsible for the assimilation of small quantities of nitrogen.

||Planting the soil somewhat reduces volatilization, most probably because the plant assimilates part of the nitrogen in its different inorganic forms before the compounds can react with each other. In one respect this experiment is important as in this case no organic nitrogen has been ammonified, which proves that considerable volatilization can take place independently of ammonification.

TABLE XV

NITROGEN BALANCE OF THE SAME SOIL, BUT INFECTED WITH 2% GARDEN SOIL, WITH AND WITHOUT CaCO<sub>3</sub> ADDED, AND PLANTED AND NOT PLANTED WITH *Brassica napus* (MG. N/100 G. DRY SOIL)

Time, days	NH <sub>4</sub> <sup>+</sup> -N	NO <sub>3</sub> <sup>-</sup> +NO <sub>2</sub> <sup>-</sup> -N	Organic N	Total N	Total N lost		Organic N mineral- ized	N in plants	pH
					Mg.	% NH <sub>4</sub> <sup>+</sup> -N added			
Expt. 2A—soil + 2% garden soil, no CaCO <sub>3</sub>									
0	43.0	2.2	98.9	144.1	—	—	—	—	5.1
25	31.1	5.4	97.0	133.5	10.6	23	1.9	—	4.9
43	31.4	11.4	87.0	129.8	14.3	33	11.9	—	4.6
66	26.6	13.1	85.0	124.7	19.4	45	13.9	—	4.3
80	18.7	10.7	81.0	110.4	33.7	78	18.9	—	4.1
Expt. 2B—as 2A + 15.9 g. of CaCO <sub>3</sub> per kg. of soil									
0	43.0	2.2	98.9	144.1	—	—	—	—	6.8
25	9.8	19.3	115.9	145.0	0	—	-17.0	—	5.7
43	3.4	30.4	111.0	144.8	0	—	-12.1	—	5.8
66	4.1	24.6	115.0	143.7	0	—	-18.1	—	5.7
80	1.4	34.7	110.0	146.1	0	—	-11.1	—	5.2
Mean 144.7									
Expt. 2C—as 2A, but planted with <i>Brassica napus</i>									
0	43.0	2.2	98.9	144.1	—	—	—	—	5.1
30	32.0	5.8	95.0	133.5	10.6	23	3.9	0.8	4.7
50	13.8	8.0	90.2	119.8	24.3	53	8.7	7.8	4.4
73	0.9	0.1	81.0	117.3	26.8	59	17.9	35.3	4.0

TABLE XVI

NITROGEN BALANCE OF THE SAME SOIL, DRESSED WITH SODIUM NITRATE INSTEAD OF AMMONIUM SULPHATE (MG. N/100 G. DRY SOIL)

Time, days	NH <sub>4</sub> <sup>+</sup> -N	NO <sub>2</sub> <sup>-</sup> + NO <sub>3</sub> <sup>-</sup> -N	Organic N	Total N	Total N lost		Organic N	pH
					Mg.	% NO <sub>2</sub> <sup>-</sup> -N added		
Expt. 3								
0	1.8	42.4	98.9	143.1	—	—	—	4.9
30	2.3	43.7	97.0	143.0	—	—	1.9	4.9
43	0.2	43.0	101.0	143.0	—	—	-2.1	5.1
59	0.9	40.0	102.0	142.9	—	—	-3.1	4.8
87	0.6	41.3	101.0	142.6	—	—	-2.1	4.6
			Mean	142.9				

plant has assimilated 24.5 mg. of N, exhausting the supply both of ammonia and nitrite + nitrate-N (12.9 + 7.9 mg.) and even part of the nitrogen set free by mineralization, leaving only 2.5 mg. for volatilization.

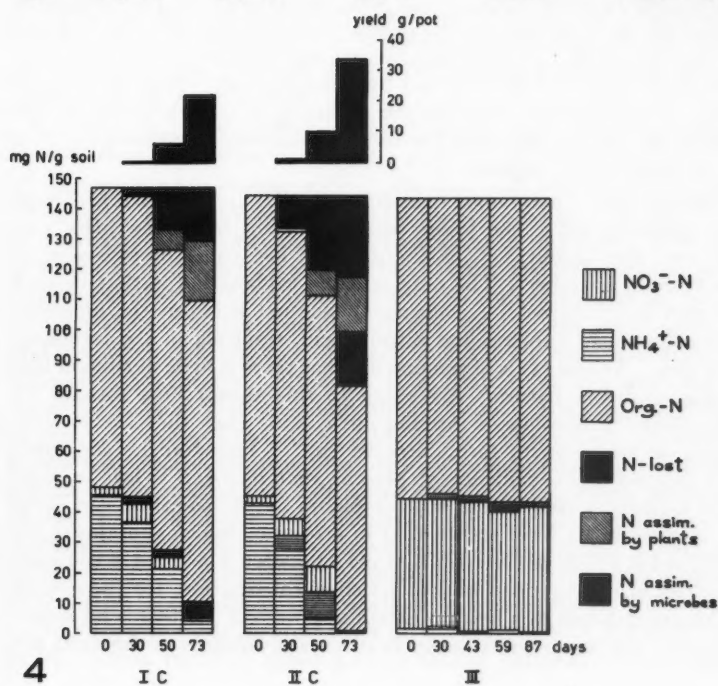
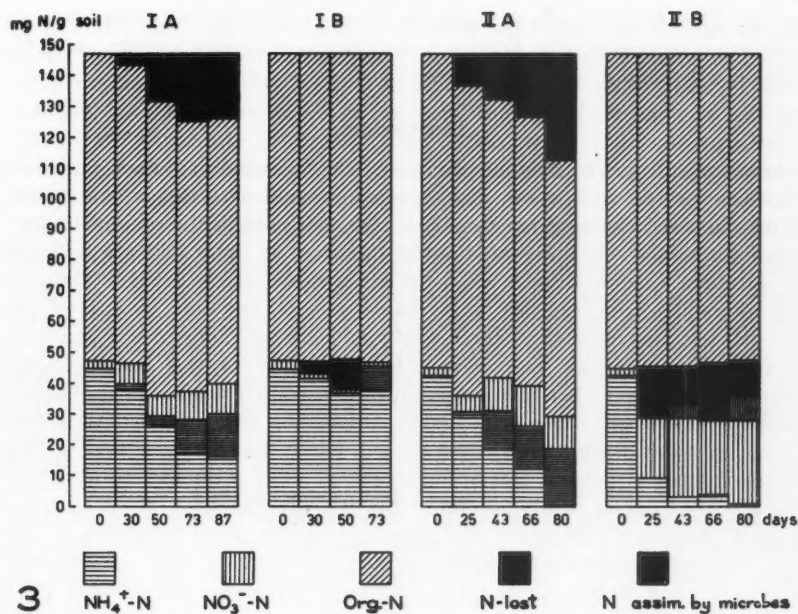
In this experiment no nitrogen losses are observed and it simply proves that denitrification as a cause for volatilization can be ruled out completely under the circumstances of these experiments.\* In Figs. 3 and 4 the results of these experiments have been indicated graphically. The nitrogen which is derived from the soil organic matter by ammonification has been hatched doubly; for instance, in 1A after 87 days nearly half of the ammonia nitrogen found in the soil originated from the organic material; in 2B after 80 days the same is the case with about one-fourth of the nitrate. It is evident that in both 1A and 2A volatilization coincides with considerable ammonification. Fig. 1B shows that when the nitrifying bacteria have been eliminated by pasteurization at 75° C. the remaining microorganisms ammonify part of the organic nitrogen, but are unable to induce volatilization, proving once more that both processes can take place independently of each other. In the planted pots 1C and 2C considerable volatilization is observed without any ammonification being detectable. It is clear however that in 2C nearly half of the nitrogen assimilated by the plants originates from the soil organic matter and must have passed through the ammonification stage. Notwithstanding this, there is hardly any increase in volatilization between the 50th

\*In this connection the question may be asked whether nitrogen might have escaped as nitrous oxide (N<sub>2</sub>O) as has been observed by Arnold (3). According to this author no detectable release of nitrous oxide was encountered in well-aerated soils, even at high moisture contents. As in all the experiments of this investigation the moisture content was far below saturation, the aeration was optimal, no volatilization occurred when nitrate was applied instead of ammonium sulphate, and no nitrogen was lost above pH 5.5; therefore gaseous escape of nitrogen as nitrous oxide is out of the question.

FIG. 3. Graphical representation of the quantitative distribution of the different forms in which nitrogen was present in successive periods in the soil of the experiment of page 374. 1A. Original soil. 1B. The same pasteurized. 2A. The soil infected with 2% garden soil. 2B. The same as 2A, but the pH raised from 5.1 to 6.8, by the addition of CaCO<sub>3</sub>. (Hatched doubly indicates that the NH<sub>4</sub><sup>+</sup> or NO<sub>3</sub><sup>-</sup> originates from the soil organic matter.)

FIG. 4. The same graphical representation as in Fig. 3, but in this case 1C is the original soil planted with *Brassica napus*, 2C the same as 1C, but the soil infected with 2% garden soil. (About half of the nitrogen assimilated by the plants originated from the soil organic matter.) 3. The soil dressed with KNO<sub>3</sub> instead of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>.





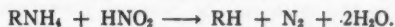
and the 73rd day. Finally attention is drawn to the fact that when nitrogen is added as nitrate (3, Fig. 4) there is hardly any microbial activity, save for a negligible amount of nitrogen assimilated by microbes.

### Discussion

Losses of nitrogen in soils are possible by the following pathways: ammonia volatilization, denitrification, and as nitrous oxide or nitrogen gas by the above discussed chemical reactions. In the soils used in the present investigation ammonia volatilization is out of question, as these soils are acid and the nitrogen losses disappear when the pH is raised above 5.5-6. The conditions for denitrification are unfavorable as well, not only because of the low pH, but also because all these sandy soils are well aerated and generally do not contain organic matter in a form suitable for this bacteriological process. Moreover when nitrogen is added as nitrate instead of as ammonium sulphate no nitrogen losses occur. The supposition that nitrogen losses were caused by volatilization of nitrogen as NO, which had been oxidized to NO<sub>2</sub> by the oxygen of the air, was at first fostered when a distinct odor of NO<sub>2</sub> was observed during sampling one of the pot experiments. By passing air through nitrifying cultures it was proved, however, that only 25% of the total nitrogen lost could be absorbed by alkaline permanganate and consisted of NO. When sterile nitrite-containing buffer solutions of different pH's were aerated, 100% of the nitrogen lost was recovered in the permanganate, proving that the method used for the determination of the NO and NO<sub>2</sub> was reliable. Consequently in nitrifying cultures the nitrogen was mainly lost in a form which could not be absorbed by permanganate, either as nitrogen or as N<sub>2</sub>O. The difference between the nitrifying culture and the nitrite-containing buffer solution was that the former contained a certain amount of ammonia as ammonium sulphate.

When sterile buffer solutions, containing KNO<sub>2</sub> and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, were aerated at different pH's, the losses increased with decreasing pH's and, again, no more than about 30% of the nitrogen lost could be recovered in the permanganate scrubber. The only possible explanation is that in both cases nitrogen is volatilized by a chemical reaction between the ammonia present as ammonium sulphate and nitrous acid, resulting in the formation of nitrogen gas. Allison (1) is of the opinion that there is little likelihood that this reaction occurs in nature. According to this author "the nitrites are much more likely to decompose forming nitric oxide than they are to react with amines or ammonia to form nitrogen gas". Therefore he concluded that there is little or no evidence at present that nitrogen is lost in more than minor quantities from either soils or plants by the so-called van Slyke reaction. However, we have shown that in a solution which contains 0.3% N as KNO<sub>2</sub> and 0.4% N as (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> at pH's between 5.5 and 4.0 considerable quantities of nitrogen can be lost by chemical reaction, for the greater part as nitrogen gas and to a smaller extent also as NO. There is no reason to suppose that the losses which occur in nitrifying cultures in the same pH

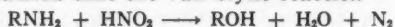
range and at about the same concentrations of  $\text{NH}_4^+$  and  $\text{NO}_2^-$  are not brought about by the same reaction. These ions do not react with each other unless the pH is below 5.5. As nitrous acid ( $\text{HNO}_2$ ) has a low dissociation constant, the  $\text{NO}_2^-$  ion added as  $\text{KNO}_2$  or produced by the nitrite bacteria will give rise to the formation of undissociated  $\text{HNO}_2$  when the concentration of hydrogen ions is raised, and it is this compound which reacts with the ammonia compounds according to the reaction



Consequently volatilization of nitrogen in liquid cultures of nitrifying bacteria is simply conditioned by a favorable buffer capacity of the solution, which allows the pH to decrease during the process of nitrification from about 6 or 5.5 to between 5.5 and 4, provided that the solution contains sufficient ammonium sulphate to keep up a certain concentration of  $\text{NH}_4^+$  ions and that the nitrifying bacteria are in an active state. In soils, conditions favoring volatilization are in broad outline the same, although in several cases at an initial pH of the soil of about 4.5 to 4.0 considerable volatilization was observed in the original soil without any addition (Tables X, XII, XIII). Thus it is not always necessary that a whole series of decreasing pH's be traversed before the above reaction becomes apparent. In other cases where the soil in its original state did not show volatilization, the addition of a small amount of  $\text{CaCO}_3$  could change the pH and the buffer capacity in such a manner that the pH became favorable for the nitrifying bacteria to start nitrification at a reasonable rate, whereas the buffer capacity allowed the pH of the soil to drop gradually during nitrification below about 5.5, which is conditional for volatilization.

The fact that the nitrifying bacteria in one soil are better adapted to a low pH than in another, as has been observed by several authors (6), is one of the causes why in some soils nitrification and volatilization proceed at a pH of about 4.5 and 4, whilst in other cases much higher pH's are necessary. In two cases out of 14 (only one is mentioned in this paper, Table XI) volatilization of N did not occur after the pH and the buffer capacity had been made optimal by the addition of  $\text{CaCO}_3$ . In both cases however the nitrifying bacteria showed a much smaller activity than in the other soils; this and perhaps other still unknown reasons might have been the explanation of these two exceptions. Although volatilization of nitrogen can proceed quite independently of ammonification, in many cases both processes seem to coincide (Tables VII, IX, X, XII, XIII). There might, however, be one reason why volatilization is promoted by ammonification. Part of the soil organic substances which fall a victim to ammonifying microorganisms consist of the dead bodies of bacteria, fungi, and actinomycetes containing proteins. During this process of microbial decay a great variety of amino acids is produced as intermediary products, and at the pH's which happen to occur in these soils at the period of volatilization (4.5 Table VII, 4.4 Table VII, 4.0 Table X, etc.) these products react with the  $\text{HNO}_2$  produced by the nitrite bacteria, opening up a second pathway for volatilization of

nitrogen as nitrogen gas, as was already shown by Allison and Doetsch (2). They have demonstrated that the van Slyke reaction



proceeds to a measurable degree at a pH of 4.5; in 24 hours 2.5% of the nitrite added reacted with alanine. It seems, however, that these authors underestimated the importance of this reaction as the percentage of the nitrogen evolved might be much higher when the process lasts several weeks instead of hours, as is the case in the present investigation. One might consider whether volatilization of nitrogen, as described in this paper, can be of such a magnitude that it plays a role in practical agriculture. In general, dressings of ammonium sulphate are not applied to acid sandy soils, but ammonification might be an important source of ammonia in some cases. In the present investigation it has happened more than once (Tables IX and X) that nearly all the nitrogen that had been volatilized originated from the ammonified organic nitrogen originally present in the soil. In this connection it seems worthwhile to mention that in ordering the soil samples for these experiments to be taken in different parts of the country, only the following requirements were made: acid sandy soils of a pH between 4 and 6, with moderate humus content. The result was that of the 14 soils received, after having been dressed with ammonium sulphate, nine showed volatilization in their original state, three did so after adding a small amount of  $\text{CaCO}_3$ , and only in two cases were no losses observed. It is evident that in soils of this type volatilization is a common phenomenon. Surveying the numerous cases of field and lysimeter experiments mentioned in the literature (1), where the nitrogen balance shows an important deficit, it is highly probable that at least in acid sandy soils volatilization of nitrogen, as nitrous oxide and as nitrogen gas, is closely related to the bacteriological processes of ammonification and denitrification, which processes in combination with the chemical reactions between nitrous acid and ammonia or amino acids play an important and unexpected role in these nitrogen losses.

#### Acknowledgment

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## THE INFLUENCE OF BORON ON THE MORPHOLOGY OF SERRATIA MARCESCENS AND ON ITS PRODUCTION OF CHOLINE PHOSPHATASE<sup>1</sup>

C. ARNAUDI AND GIULIANA NOVATI

### Abstract

A strain of *Serratia marcescens* with marked lecithinase action has been isolated. It causes a selective hydrolysis of egg lecithin. In order to obtain the hydrolysis due to the choline phosphatase it is necessary to buffer the egg-lecithin emulsion at pH 8.8 with sodium tetraborate. Sodium tetraborate in quantities corresponding to 1.91% inhibits the lecithinase A and the lecithinase B of *S. marcescens* but not the activity of choline phosphatase. Choline phosphatase appears to be an endoenzyme and has been extracted from bacterial cells by means of water and toluene, and by means of ultrasonic disintegration. The inactivation of the enzyme by heat and the action of toluene on the enzyme have been studied. Boron in culture media causes morphological modifications of *S. marcescens*, which may appear as filaments. Thallium, gallium, and aluminum produce no morphological modifications.

### Introduction

It is well known that enzyme preparations from animal and plant sources can cause the hydrolysis of lecithin. The end products of hydrolysis differ according to the point of enzyme action on the lecithin molecule. There are four possible points that can be attacked; the two linkages between glycerol and fatty acids, the linkage between glycerol and phosphoric acid, and the phosphoric acid-choline linkage. The enzymes which can act at these points are:

*Lecithinase A*—which can split off the unsaturated fatty acid giving rise to lysolecithin, which has a high hemolytic activity;

*Lecithinase B*—which can split off from lecithin the two molecules of fatty acids and from lysolecithin the remaining molecule of fatty acid giving rise to glyceryl-phosphorylcholine;

*Lecithinase C*—which acts on the linkage between choline and phosphoric acid liberating phosphatidic acid and choline; and

*Lecithinase D*—by which the hydrolysis of the linkage between glycerol and phosphoric acid is obtained with the production of phosphorylcholine and a diglyceride.

The best known of all the above-mentioned enzymes is without doubt lecithinase A. It has been found in the venom of some snakes, in wasp poison, and in the pancreas of some mammalia (see Delezenne, Fournan, Ledebt, Belfanti, Contardi, Arnaudi, Ercoli, Lotzer, etc., reported from Desnuelles (5) and from Wittcoff (26)). Much less is known of the other lecithinases which have been found in the tissues of some plants and in some

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species of eumycetes and schizomycetes. We cannot affirm that lecithinase A and lecithinase B are two different enzymes on the sole basis that the hydrolysis which they produce takes place at two different values of pH 6.8-7.3 for lecithinase A and of pH 3.5 for lecithinase B. Most probably it is a single enzyme, which acts at two different values of pH. The terms lecithinase A and lecithinase B are differently employed by various authors. Desnuelles (5) names lecithinase D the enzyme that we have reported as lecithinase C and vice versa. Quantitative criteria are of extreme importance for the assessment of the products of hydrolysis. Recently Jacini and Arnaudi (14), experimenting on the lecithinase of *S. marcescens*, demonstrated that during the course of the hydrolysis at pH 8.8 in the presence of a borate buffer there is an inhibition of the formation of lysolecithin, while 90% of the choline is liberated. We think that one of the other parts that have been separated contains more than 50% of phosphatidic acids. In fact this part contains up to 2.3% of phosphorus and is free from choline and water-soluble substances. During the course of this research boron appeared to possess a specific action on the elimination of the lecithinase C produced by *S. marcescens*. This has been reported by Arnaudi and Novati (1), who proved that boron interferes in the metabolism of the microorganism and in the action of the enzyme obtained from the organism.

As early as 1901, Ruata and Caneva (25) found that *S. marcescens* has an enzymatic activity on lecithin. They referred the hydrolytic action of lecithin to the metabolic process involved in the multiplication of bacteria, as they could not prove the presence of the enzyme in the cultures. They were the first to employ the Florence reagent for the determination of the free choline, and they were able to demonstrate the splitting activity of various schizomycetes, among which there was *S. marcescens*. Monsour and Colmer (19) have recently employed the appearance of free choline in agar cultures in the presence of lecithin as a criterion for the classification of the different strains of *S. marcescens*.

### Materials and Methods

1. We have isolated a strain of *S. marcescens* (strain I3), which has lecithinase A, B, and C activities.
2. *S. marcescens* grown on nutrient agar (Difco) was inoculated into egg-yolk and egg-lecithin emulsions.
3. The egg-yolk emulsions were prepared by adding egg yolks to 100 ml. of sterile physiological saline.
4. The egg-lecithin emulsion was prepared, unless otherwise specified, by adding 5% of egg lecithin (egg lecithin ERBA) to a sterile buffer containing 5 ml. of monopotassium phosphate *M*/10 and 95 ml. of sodium tetraborate *M*/10 (pH 9.2). The medium was agitated and when the emulsion was obtained the pH was 8.8.
5. Lecithinase A was detected by studying the hemolysis of sheep's red blood cells in test tubes. The test tubes were prepared as follows: 1 ml. of



sterile physiological solution + 0.5 ml. of a 5% suspension of sheep's red blood cells in physiological solution + 0.5 ml. of egg-yolk or egg-lecithin emulsion inoculated with *S. marcescens*. Controls were prepared with emulsions which had not been inoculated with *S. marcescens*. The red blood cells were controlled by the addition of 1 ml. of physiological saline to 1 ml. of the suspension of red blood cells. The test tubes were kept from 14 to 18 hours at 37° C. and for a few hours at 2° C. before the results were checked.

6. Lecithinase B activity was detected by recording the increase of the acidity of the medium.

7. The activity of the lecithinase C and the activity of the choline phosphatase have been detected by means of the Florence reagent, which precipitates choline as crystals of periodide, which can be seen through the microscope.

### Results

The Florence reaction was positive 48 hours after *S. marcescens* I3 had been inoculated into the egg-yolk emulsions and incubated at 30° C. The pH was greatly diminished and the hemolysis test was positive. In addition to the choline phosphatase activity there was a lecithinase A and a lecithinase B activity. We have buffered the egg-yolk emulsions in order to impede the lecithinase A and B activities without inhibiting choline phosphatase. The following buffers were employed:

1. Disodium phosphate *M*/15 and potassium phosphate *M*/15 in ratios to give pH 5.2, 5.6, 6.2, 6.8;

2. Citric acid *M*/10 and disodium phosphate *M*/5 in ratios to give pH 4.2, 4.8, 6.7;

3. Succinic acid *M*/20 and sodium tetraborate *M*/20 in ratios to give pH 4.2, 4.8;

4. Sodium hydrate *M*/10 and glycine *M*/10 in ratios to give pH 8.6.

In all these emulsions, after they had been inoculated with *S. marcescens* and incubated at 30° C. and at 37° C., lysolecithin and fatty acids, in addition to choline, were detected. We were not able to detect choline in controls and the hemolysis test was negative. Choline could be detected after 6-8 days by the addition of egg yolks to a buffer containing potassium phosphate *M*/10 and sodium tetraborate *M*/10 of pH 9.2 (pH 8.8 after the egg yolk had been added) and by the inoculation into it of *S. marcescens*. The hemolysis test was negative. Choline could be detected after an incubation of only 28 hours by adding a 5% emulsion of egg lecithin in the same buffer. So, egg lecithin instead of egg yolk has been employed as a medium during the course of this research.

Quantitative analysis showed that choline increased in proportion to the length of incubation.\* The percentages of choline which have been obtained were: 24% after 5 days; 27% after 10 days; 29% after 15 days; and 33% after 20 days. The hemolysis test always remained negative when the

\*Quantitative analysis has been performed by Professor Jacini, of the Stazione Sperimentale Olii e Grassi, Milan, Italy.

emulsions were incubated at 30° and at 37° C. Twenty days after *S. marcescens* had been inoculated into physiological solution, containing a 5% emulsion of egg lecithin, only 15% of choline in addition to lysolecithin and fatty acids was detected. The activity of the lecithinase A and of the lecithinase B was not detectable by means of the tetraborate buffer, while choline phosphatase was increased.

We have also studied the action of pH and sodium tetraborate on the hydrolysis of the egg lecithin. Egg lecithin was added to the sodium tetraborate buffer in order to obtain emulsions of pH from 4.5 to 8.8. *S. marcescens* was inoculated into each emulsion which was then incubated at 30° C. A part of each emulsion was kept for control. The appearance of free choline and hemolytic activity was followed and the pH controlled. The results have been compared to those obtained by means of emulsions of egg lecithin in phosphate buffer inoculated in the same manner as the tetraborate emulsions. The two groups of emulsions had almost the same pH (Tables I and II).

A pH higher than 7.4 could not be obtained by means of the phosphate buffer. NaOH was added to physiological solution containing 5% of egg lecithin in order to obtain pH 8 and pH 8.8. Table I shows that choline could be detected in the egg-lecithin emulsion in phosphate buffer, but the hemolysis test was positive with partial agglutination of the red blood cells and with a decrease of the pH, notwithstanding the starting values.

Table II shows that choline could be detected in borate buffer of pH from 4.6 to 5.8. The hemolysis test was positive and there was agglutination. Only at pH 8.7-8.8 did choline appear rapidly, while there were no modifications of the pH, no hemolysis, and no agglutination. As all controls showed no choline or hemolysis or agglutination or modification of the pH, the transformation of lecithin into hemolytic lysolecithin and the formation of choline and fatty acids were due to the activity of *S. marcescens*. The action of the lecithinase A appeared before that of the choline phosphatase because when there was hemolysis it appeared before choline was detectable. The activity of lecithinase A remained when choline phosphatase had become active, because in several cases hemolysis increased after choline started to be detectable. Since, in the emulsions adjusted to pH 8 and 8.8 with NaOH, choline and also lysolecithin were detectable while the pH decreased, it was clear that sodium tetraborate and not the pH inhibited lecithinase A and lecithinase B. In fact, tetraborate was present in small quantities (Table II) in the emulsions in which choline was detected and the hemolysis test was positive. In the emulsions in which choline was detected but the hemolysis test was negative, tetraborate (from 1.6 to 1.9%) and not the pH was active.

#### *Extraction of Choline Phosphatase*

We have already mentioned that the choline phosphatase of *S. marcescens* behaves as an endoenzyme. This enzyme acts in the presence of sodium tetraborate, which causes severe modifications of the metabolism of the organism leading to its death. Moreover it has been observed that filtrates of broth cultures of 20-25 days' incubation had choline phosphatase properties.

TABLE I  
THE ACTION OF *Serratia marcescens* ON THE LECITHIN OF EGG YOLK IN PHOSPHATE BUFFER

	Days										
	2	5	7	9	11	13	20	Since 7th day			
pH 4.6	Choline Hemolysis pH ++--	-	+++	+	+	++	++ 4.2	Partial agglutination*			
Contr.†	Hemolysis pH +---		+---				++ 4.4	No agglutination			
pH 4.8	Choline Hemolysis pH ++--	-	+++	+	+	++	++ 4.4	Partial agglutination*			
Contr.†	Hemolysis pH +---		+---				++ 4.5	No agglutination			
pH 5	Choline Hemolysis pH ++--	-	+++	+	+	++	++ 4.4	Partial agglutination*			
Contr.†	Hemolysis pH +---		+---				++ 4.6	No agglutination			
pH 5.2	Choline Hemolysis pH ++--	+	+++	+	+	++	++ 4.6	Partial agglutination*			
Contr.†	Hemolysis pH +---		+---				++ 4.8	No agglutination			
pH 5.4	Hemolysis Choline pH ++--	+	+++	+	+	++	++ 4.8	Partial agglutination*			
Contr.†	Hemolysis pH +---		+---				++ 5	No agglutination			
pH 5.6	Choline Hemolysis pH ++--	+	+++	+	+	++	++ 4.6	Partial agglutination*			
Contr.†	Hemolysis pH +---		+---				++ 5	No agglutination			

\*Superficial coagulum, layering of fatty acids.

†No choline.

TABLE I—*Concluded*  
THE ACTION OF *Serratia marcescens* ON THE LECITHIN OF EGG YOLK IN PHOSPHATE BUFFER—*Concluded*

	Days										
	2	5	7	9	11	13	20	Since 7th day			
pH 5.7	Choline Hemolysis pH	++--	-	+++-	+	++	++ 5.2	Partial agglutination*			
Contr. †	Hemolysis pH	++--	++--	++--			+- 5.4	No agglutination			
pH 6	Choline Hemolysis pH	++--	-	+++-	-	++	++ 5.5	Partial agglutination			
Contr. †	Hemolysis pH	++--	++--	++--			+- 5.7	No agglutination			
pH 6.4	Choline Hemolysis pH	++--	+	+++-	+	++	++ 5.8	Partial agglutination			
Contr. †	Hemolysis pH	++--	++--	++--			+- 6.4	No agglutination			
pH 6.8	Choline Hemolysis pH	++--	+	+++-	+	++	++ 5.9	Partial agglutination			
Contr. †	Hemolysis pH	++--	++--	++--			+- 6.6	No agglutination			
pH 7.4	Choline Hemolysis pH	++--	+	+++-	+	++	++ 6	Partial agglutination			
Contr. †	Hemolysis pH	++--	++--	++--			+- 6.7	No agglutination			

\*Superficial coagulum, layering of fatty acids.

†No choline.

TABLE II  
THE ACTION OF *Serratia marcescens* ON THE LECITHIN OF EGG YOLK BUFFERED WITH SODIUM TETRABORATE BUFFER

Days		2	5	7	8	9	11	13	20	20th day
Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub> : 0.05 g. % pH 4.6 Contr. ‡	Choline Hemolysis pH	+++-	+	+++	++	++	++	+++	+++ 4.4	Agglutination*
	Hemolysis pH	+-	+	+	++	++	++	++	+- 4.4	No agglutination*
Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub> : 0.10 g. % pH 4.8 Contr. ‡	Choline Hemolysis pH	+++-	+	+++	++	++	++	+++	+++ 4.4	Agglutination*
	Hemolysis pH	+-	+	+	++	++	++	++	+- 4.6	No agglutination*
Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub> : 0.20 g. % pH 5.2 Contr. ‡	Choline Hemolysis pH	+++-	+	+++	+	+	+	+	+++ 4.6	Agglutination*
	Hemolysis pH	+-	+	+	++	++	++	++	+- 5.1	No agglutination*
Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub> : 0.25 g. % pH 5.4 Contr. ‡	Choline Hemolysis pH	+++-	-	+++	-	+	+	+	+++ 5	Partial agglutination*
	Hemolysis pH	+-	+	+	++	++	++	++	+- 5.3	No agglutination*
Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub> : 0.30 g. % pH 5.6 Contr. ‡	Choline Hemolysis pH	+++-	-	+++	+	+	+	+	+++ 5.5	No agglutination*
	Hemolysis pH	+-	+	+	++	++	++	++	+- 5.6	No agglutination*
Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub> : 0.40 g. % pH 5.7 Contr. ‡	Choline Hemolysis pH	+++-	-	+++	+	+	+	+	+++ 5.6	No agglutination†
	Hemolysis pH	+-	+	+	++	++	++	++	+- 5.7	No agglutination

\*Almost complete coagulation, layering of fatty acids.

†Superficial coagulum, layering of fatty acids.

‡No choline.

TABLE II—*Concluded*  
 THE ACTION OF *Serratia marcescens* ON THE LECITHIN OF EGG YOLK BUFFERED WITH SODIUM TETRABORATE BUFFER—*Concluded*

	Days										20th day
	2	5	7	8	9	11	13	20			
Choline Hemolysis pH	+	-	-	-	-	+	+	+	No agglutination†		
Hemolysis pH	+	-	-	-	-	-	-	+	No agglutination		
Choline Hemolysis pH	-	-	-	-	-	-	-	+	No agglutination		
Hemolysis pH	+	-	+	-	-	-	-	+	No agglutination		
Choline Hemolysis pH	+	-	-	-	+	+	+	+	No agglutination		
Hemolysis pH	+	-	+	-	-	-	-	+	No agglutination		
Choline Hemolysis pH	+	+	+	+	+	+	+	+	No agglutination		
Hemolysis pH	+	-	+	+	+	+	+	+	No agglutination		
Choline Hemolysis pH	+	+	+	+	+	+	+	+	No agglutination		
Hemolysis pH	+	-	+	+	+	+	+	+	No agglutination		
Choline Hemolysis pH	+	+	+	+	+	+	+	+	No agglutination		
Hemolysis pH	+	-	+	+	+	+	+	+	No agglutination		
Choline Hemolysis pH	+	+	+	+	+	+	+	+	No agglutination		
Hemolysis pH	+	-	+	+	+	+	+	+	No agglutination		

\*Almost complete coagulation, layering of fatty acids.

†Superficial coagulum, layering of fatty acids.

‡No choline.



Thus the enzyme has been shown to be present in the culture medium as a consequence of the destruction of cells. The high sodium tetraborate concentration (1.92%) of the egg-lecithin emulsion accelerated this process. Several attempts have been made in order to extract choline phosphatase from cells. The extraction by means of alumina according to McIlwain's method (18) did not give good results. Two methods for the extraction of choline phosphatase from *S. marcescens* have given fairly good results. First of all, the growth medium is of great importance for the production of choline phosphatase. The cells that have been cultivated on egg-lecithin agar (nutrient broth (Difco), 0.8%: egg lecithin 1%: agar-agar 2%) liberated choline quicker than the cells cultivated on normal agar without egg lecithin (Table III). The age of the cultures was of importance and 8-day cultures yielded media with higher enzymatic activities in comparison with younger cultures. The importance was less clear when the cells were inoculated into egg-lecithin emulsions and even 48-day-old cultures still had choline phosphatase properties (Table IV).

TABLE III  
PRODUCTION OF CHOLINE FROM LECITHIN OF EGG YOLK

	Time, hr.	
	24	30
Sterile egg-yolk lecithin (control)	—	—
Lecithin inoculated with <i>S. marcescens</i> grown on nutrient agar	—	+
Lecithin inoculated with <i>S. marcescens</i> grown on nutrient agar + 1% lecithin	+	+

TABLE IV  
PRODUCTION OF CHOLINE FROM LECITHIN OF EGG YOLK

	Time, hr.		
	24	72	96
Sterile egg-yolk lecithin (control)	—	—	—
Lecithin inoculated with <i>S. marcescens</i> (1-day culture)	+	++	+++
Lecithin inoculated with <i>S. marcescens</i> (3-day culture)	+	+++	+++
Lecithin inoculated with <i>S. marcescens</i> (8-day culture)	+	+++	+++
Lecithin inoculated with <i>S. marcescens</i> (48-day culture)	—	++	+++

#### *Methods Employed for the Extraction of Choline Phosphatase*

(A) After an incubation of 8 days the cells were collected in distilled water to which 5% toluene was added. The suspension of the cells was kept 8 days at 30° C. and then was filtered through a Richard-Ginori porcelain filter and then concentrated in a vacuum. The sterile liquid obtained had a pH of 6.8 and had choline phosphatase activity.

(B) After an incubation of 8 days, the cells were collected in distilled water and, in quantities of 50 ml., without the addition of toluene or of other antiseptics, were treated with ultrasonics for 15 minutes. A frequency of 599 kHz. and a power of 0.8 w./sq. cm. at 1200 v. were employed. The suspension was then filtered and the filtrate concentrated in a vacuum. The sterile liquid obtained had a pH of 6.8 and, added to sterile suspensions of egg lecithin, liberated choline in a period of from 48 hours to 3 days. In order to obtain the maximum enzymatic activity all the phases of the extraction process should not exceed 2-3 days.

The number of the cells remaining in the suspensions after ultrasonic treatment proved to be only 50% of the starting value. By means of the electron microscope, normal cells and cell membranes, with only part of their content or completely emptied, could be seen (see Fig. 1). The power and ultrasonic frequency employed, generally sufficient to kill bacterial cultures (22), were not able to disintegrate the bacterial cells (3). The number of the cells disintegrated increased as the treatment with ultrasonics was prolonged to 30 minutes. Yet the enzymatic activity of the medium did not increase (Table V).

TABLE V  
PRODUCTION OF CHOLINE FROM LECITHIN OF EGG YOLK

	Time, hr.	
	48	72
Sterile egg-yolk lecithin (control)	—	—
Lecithin inoculated with <i>S. marcescens</i>	+	++
Lecithin + enzyme (extracted with distilled water and toluene)	+	++
Lecithin + enzyme (extracted by 15 min. treatment in sonic oscillator)	+	++
Lecithin + enzyme (extracted by 30 min. treatment in sonic oscillator)	+	++

*The Properties of the Choline Phosphatase Isolated from Serratia marcescens*

The extract obtained from *S. marcescens* could split choline from egg lecithin, but did not lower the pH. Thus it contained only choline phosphatase, which was water-soluble and, in aqueous solution of pH 6.8, could be filtered without adsorption by the filter. This choline phosphatase was inactivated in 5 minutes at 70° C. (pH 6.8), while in aqueous solution of 18-20° C. remained active for several months. This choline phosphatase maintains its activity for a long time when lyophilized. This enzyme can be precipitated by acetone from aqueous solutions. The precipitate appears as a brownish, sticky mass and its choline phosphatase activity is a little reduced as if acetone were responsible for the partial inactivation of the enzyme (Table VI).

*Action of Toluene on Choline Phosphatase*

Choline phosphatase, as we have already mentioned, can be isolated by leaving the cells in distilled water containing 5% of toluene. The studies of Hanahan (12) on the activation of lecithinase A by ether and the studies of

TABLE VI  
PRODUCTION OF CHOLINE FROM LECITHIN OF EGG YOLK

	Time, days		
	3	10	15
Sterile egg-yolk lecithin (control)	—	—	—
Lecithin + cell-free extract 0.5 ml.	+	++	++
Lecithin + acetone precipitated enzyme from 0.5 ml. of cell-free extract	+	+	+

Kates (15) on the activation of the choline phosphatase of plastids by ether suggest a possible effect of toluene on choline phosphatase. Thus a sterile suspension of egg lecithin was prepared and stored in test tubes. To some of the tubes 5% toluene was added and 5% ether to the others. The suspension was inoculated and incubated at 30° C., while controls were not inoculated (Table VII).

TABLE VII  
PRODUCTION OF CHOLINE FROM LECITHIN OF EGG YOLK

	Time			
	18 hr.	28 hr.	116 hr.	30 days
Sterile egg-yolk lecithin (control)	—	—	—	—
Lecithin inoculated with <i>S. marcescens</i>	—	+	++	++
Sterile lecithin + toluene (control)	—	—	—	—
Lecithin + toluene inoculated with <i>S. marcescens</i>	+	++	++	++
Sterile lecithin + ether (control)	—	—	—	—
Lecithin + ether inoculated with <i>S. marcescens</i>	+	++	++	++

*S. marcescens* in the presence of ether and of toluene liberated choline quicker and in greater quantities than in the controls. Ether and toluene both favored the splitting of choline by the enzyme. Ether when added to the egg-lecithin emulsion immediately modified it. The emulsion separated into two layers: a supernatant extract containing the fatty acids and an aqueous layer. As it was necessary to keep the material at 30° C., subsequent research was limited to toluene, which has a higher boiling point (110° C.) than ether (34.6° C.). To ascertain whether toluene acts on bacterial cells (favoring the release of the enzyme from the cells) or directly on egg lecithin, 5% toluene was added to an egg-lecithin emulsion, which was then incubated at 30° C. After 24 hours the toluene was completely removed and the emulsion was transferred into clean test tubes and inoculated. Moreover *S. marcescens* was inoculated into an emulsion of normal egg lecithin and into an emulsion of egg lecithin to which 5% of toluene was added at the moment of the inoculation (Table VIII).

TABLE VIII  
PRODUCTION OF CHOLINE FROM LECITHIN OF EGG YOLK

	Time, days			
	1	2	5	20
Sterile egg-yolk lecithin (control)	—	—	—	—
Lecithin inoculated with <i>S. marcescens</i>	—	+	++	+++
Sterile lecithin + toluene (control)	—	—	—	—
Lecithin + toluene inoculated with <i>S. marcescens</i>	+	++	+++	+++
Sterile lecithin after 24 hours' contact with toluene (control)	—	—	—	—
Lecithin after 24 hours' contact with toluene + <i>S. marcescens</i>	+++	+++	+++	+++

Twenty-four hours after inoculation of the egg lecithin, which had been 24 hours in the presence of toluene, choline was detectable in quantities greater than in the emulsion to which toluene had been added at the moment of the inoculation. So, egg lecithin and not the bacterial cells had been modified by toluene. On the other hand, specimens of each emulsion which had not been inoculated with *S. marcescens* did not prove to contain any choline. Moreover, 5% toluene added to an egg-lecithin emulsion produced a more rapid splitting of choline even when the emulsion was inoculated with the enzymatic extract (Table IX). The enzymatic liquid extracted from

TABLE IX  
PRODUCTION OF CHOLINE FROM LECITHIN OF EGG YOLK

	Time, hr.		
	30	48	96
Sterile egg-yolk lecithin (control)	—	—	—
Lecithin inoculated with <i>S. marcescens</i>	—	+	++
Sterile lecithin + toluene (control)	—	—	—
Lecithin + cell-free extract	—	+	++
Lecithin + toluene + cell-free extract	+	++	++

*S. marcescens* was kept in the presence of toluene for 24 hours. Toluene was then removed and the liquid was inoculated into egg-lecithin emulsions. Controls were inoculated with normal extract. Choline appeared at the same time and in the same quantities in both the emulsions. We conclude that toluene favors a quicker splitting of choline from the egg-lecithin molecule by the choline phosphatase of *S. marcescens*. Toluene alone does not split choline, not even when left for several months in the presence of egg-lecithin emulsions. Moreover, toluene neither activates the extracted choline phosphatase nor acts on the bacterial cells. Most probably toluene acts by favoring the action of the choline phosphatase on egg lecithin.

*Action of Boron on the Morphology of Serratia marcescens*

Aberrant forms of the various bacterial species have been known for a long time. Kuhn and Sternberg (17) interpreted these anomalies as due to the action of parasites of the organism which he named "pettenkoferie". The interest in these forms did not last for long because they have been considered as degenerate forms. Study has been resumed lately, after it had been proved that antibacterial drugs produce modifications of the morphology of bacteria, as in the well-known research of Fleming *et al.* (9), who have studied the action of penicillin on *Proteus vulgaris* and other bacteria. Other authors have seen that antibiotics produce enlargement of the nucleus, nuclear division without division of the cytoplasm, vesicular formations, and the appearance of new forms which resemble filaments. These modifications may appear spontaneously in old cultures and can be produced by means of physiochemical factors. Sometimes after a few inoculations these modifications become permanent, as, for instance, has been described by Klieneberger-Nobel (16) and Dienes (6) for the *Streptobacillus moniliformis*. In several other cases aberrant forms appear for a short time and there is a rapid return to normal forms. These modifications can be induced by several chemical agents. Cancer-producing substances have been added to culture media by Parvis and Sirtori (23). For example *Escherichia coli*, when inoculated in agar media to which dichlorodiethylmethylamine has been added, appears as more or less entangled elongated filaments after only 3-5 hours. After 14 hours the cells resume their normal morphology. *Bacillus subtilis* inoculated into the same medium behaves as *P. vulgaris* with the only difference that the modifications are less marked. When ethylcarbamate is added to the medium, filaments appear sometimes with vacuoles. Also *Staphylococcus aureus* appears markedly modified. Some anionic detergents (e.g. tergitol 7-08) determine in cultures of *E. coli* the appearance of elongated and tangled filaments, which after only 6 hours have completely replaced the normal forms (2). Urethane (4) or dimethylethylamine or chloroethylamine (8) cause the appearance of filaments, granular and giant forms in the cultures of *E. coli*. Filaments may also appear when urea is added to the medium (24). These modifications of bacteria, which Nickerson (20, 21) names conversion B  $\longrightarrow$  F (Bacteria  $\longrightarrow$  Filaments), is certainly due to a modification of the ratio between the growth and the multiplication of bacteria. This phenomenon may be interpreted on the basis of Hinshelwood's (13) theory concerning the dynamics of cellular multiplication. The dynamic equilibrium between the division factor "D" and the elongation factor "L" of the cytoplasm is responsible for the regulation of the cell multiplication. The prevalence of the factor "L" on the factor "D" determines the appearance of filaments in cultures of bacteria. All these appearances seem to be due to modifications of the cell metabolism which lead to impaired syntheses of metabolites or to inhibition of enzymes. Ehrlich (7) described the appearance of filaments in cultures of a strain of *S. marcescens* to which glucose or other carbohydrates had been added. We have inoculated

*S. marcescens* into culture media containing glucose, but we were not able to observe, not even after 2 months, any modification of the morphology. Nickerson and Webb (21) have seen elongation of *S. marcescens* due to aminopterin, which, in the case of *E. coli*, has a more marked effect. We were not able to find any research concerning the action of boron on the morphology of bacteria. Only Guignard and Charrin (11) in 1888 reported the appearance of filaments in cultures of *Bacillus pyocyaneus* to which antiseptics, and among them boron, had been added.

We have studied the morphology of *S. marcescens* grown in egg-lecithin emulsions of pH 8.8, containing 1.91% of sodium tetraborate. We have inoculated *S. marcescens* in an egg-lecithin emulsion containing 2% of agar. After only 24 hours the bacteria had lost their characteristic morphology of small and slender rods and appeared as long and tangled filaments. In the liquid emulsion without agar this phenomenon was less evident. Moreover sodium tetraborate acted as an antiseptic. As a matter of fact the number of living cells of the liquid emulsion progressively decreased and after 5-6 days the mortality reached 100%. The free choline content progressively increased while the hemolysis test remained negative and the pH was unmodified. The choline phosphatase of *S. marcescens* behaved as an endoenzyme, not in relation to the bacterial metabolism. The high sodium tetraborate concentration favored the release of the enzyme from the organism. As we deemed it necessary to take care that the pH was not so high as to produce the above-mentioned filaments, we prepared the following culture media: Nutrient broth Difco 0.8%, glucose 3%, brought to pH 8.8 by means of sodium hydroxide, and nutrient broth - glucose buffered at pH 8.8 with a glycine buffer (NaOH M/10 20 ml. + glycine M/10 80 ml.). *S. marcescens* was inoculated in these media with and without agar. The morphology was observed a few hours after the inoculation and then at short intervals for 20 days. The growth was hampered by the very alkaline medium, but we were not able to observe any morphological modification. *S. marcescens* had also been inoculated into nutrient broth - glucose with and without agar, to which 5% of egg lecithin had been added (pH 7). The growth was abundant while the morphology remained normal. The colonies on agar - egg lecithin assumed the characteristic pink pigmentation, which had been lost during previous cultures on nutrient broth - agar. We were able to prove that sodium tetraborate can determine the appearance of filaments in cultures of *S. marcescens*. Moreover we have studied the concentration of sodium tetraborate and the pH values which were necessary for the appearance of filaments. To nutrient broth - glucose agar the following quantities of sodium tetraborate have been added: 500 mg. %, 250 mg. %, 100 mg. %, 50 mg. %, and 25 mg. %. The pH values of each culture medium were regulated at 7.7, 7, and 6.6. Similar media without sodium tetraborate were the controls. *S. marcescens* from 24 hour cultures was inoculated into all the media, which were then kept at 30° C. The morphology was observed a few hours after the inoculation and then at short intervals of time. *S.*



PLATE I

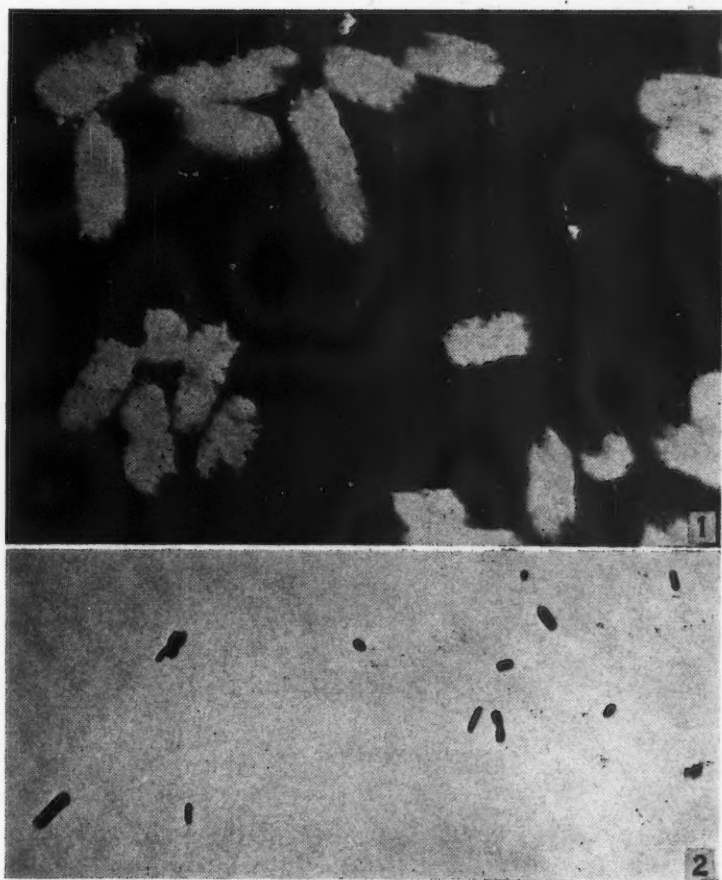


FIG. 1. Electron micrograph of *S. marcescens* after treatment in sonic oscillator (15,000X).

FIG. 2. *S. marcescens* from 48-hour nutrient glucose agar culture (control) (3000X).

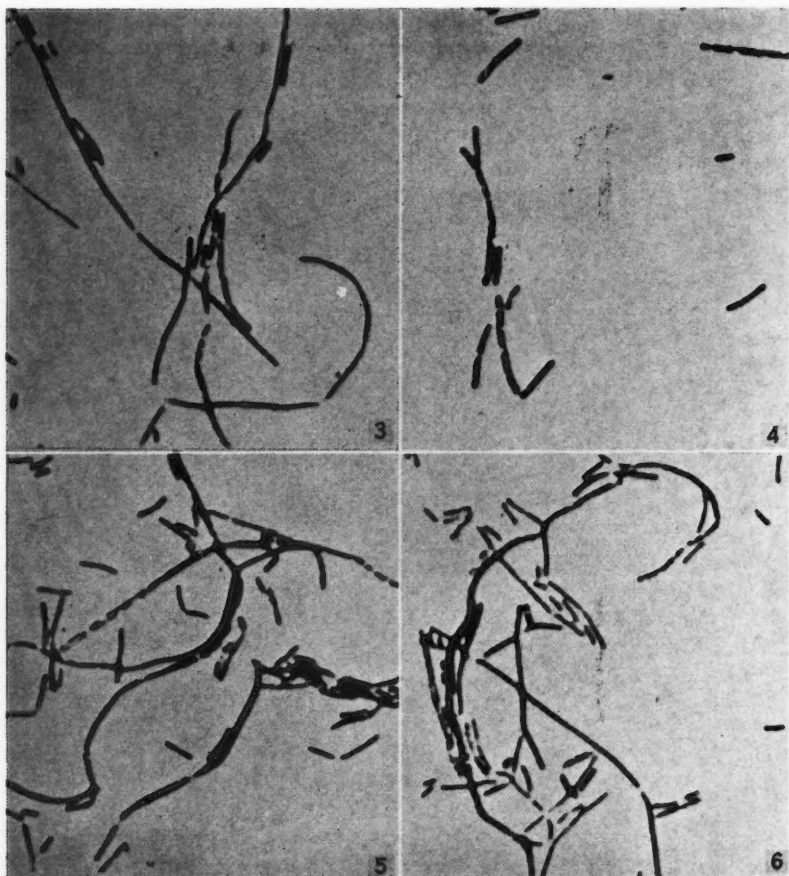


FIG. 3. *S. marcescens* from 48-hour nutrient glucose agar + 500 mg. %  $\text{Na}_2\text{B}_4\text{O}_7$  culture (2375 $\times$ ).

FIG. 4. *S. marcescens* from 10-day nutrient glucose agar + 500 mg. %  $\text{Na}_2\text{B}_4\text{O}_7$  culture (2375 $\times$ ).

FIG. 5. *S. marcescens* from 48-hour nutrient glucose agar + 250 mg. %  $\text{Na}_2\text{B}_4\text{O}_7$  culture (2375 $\times$ ).

FIG. 6. *S. marcescens* from 20-day nutrient glucose agar + 113 mg. %  $\text{NH}_4\text{HB}_4\text{O}_7 \cdot 3\text{H}_2\text{O}$ .

*marcescens* grew badly in the media containing 500 mg. % and 250 mg. % of sodium tetraborate, but 30 hours after the inoculation the bacteria already appeared as long filaments up to 20–30 times their normal length (Figs. 2 and 3). A cross wall stain (tannic acid, crystal violet, Congo red) did not show any cross walls in the filaments. Sometimes vacuoles were evident. In solid culture media at pH 7.7 filaments were much more evident, while at pH 7 and 6.6 they were also evident in liquid media.

The cells grown on media containing 100 mg. % of sodium tetraborate were only slightly longer than normal. *S. marcescens* grown on media containing 50 mg. % and 25 mg. % of sodium tetraborate and in control media did not show any morphological modification even 20 days after the inoculation. Sometimes filaments remained for as long as 20–30 days. Sometimes the filaments spontaneously disappeared after only 10 days (Fig. 4). The filaments of *S. marcescens* from a 48 hour culture on nutrient glucose agar of pH 7 (Fig. 5), to which 250 mg. % of sodium tetraborate was added, inoculated into nutrient broth–glucose agar, after only 6 hours resumed the normal appearance of short rods. The same strain did not grow when transferred in the same medium that had transformed the cells into filaments. Filaments proved to possess the same biochemical activities as rods (reduction of nitrates, liquefaction of gelatin, coagulation of milk, production of indol). Thus the filaments produced by sodium tetraborate proved to be only a morphological modification. Other boron compounds were tried in order to ascertain whether boron or only the borate was responsible. Ammonium borate ( $\text{NH}_4\text{HB}_4\text{O}_7 \cdot 3\text{H}_2\text{O}$ ) was added to the nutrient broth–glucose in quantities such as to furnish the boron contained in 250, 100, 50, and 25 mg. of sodium tetraborate respectively. *S. marcescens* was then inoculated in liquid media of pH 7.7, 7, and 6.6, containing: nutrient broth–glucose and 283, 113, 56, and 28 mg. % of ammonium borate.

In the media containing 283 mg. % of ammonium borate a greater number of long filaments was evident after an incubation of 30 hours at 30° C. After 48 hours the filaments started to break up into rods. In the media containing 113 mg. % of ammonium borate there was a greater number of filaments which were very long even after 20 days (see Fig. 6). Filaments were clearly present only in liquid media of pH 7 and 6.6. In the media with lower quantities of ammonium borate the bacteria were normal.

Culture media were prepared containing ammonium sulphate in amounts containing  $\text{NH}_4$  in quantities corresponding to those in the media with ammonium borate. *S. marcescens* inoculated in these control media did not modify its morphology. Thus it has been demonstrated that the appearance of filaments was in no relation with the content of ammonium ions of the culture media. Moreover culture media were prepared containing sodium perborate and boric acid to give quantities of boron corresponding to those of the above-mentioned media. In solid media of pH 7.7 containing 764 mg. % and 304 mg. % of sodium perborate (containing the same quantities of boron as 250 mg. and 100 mg. % respectively of sodium tetraborate)

filaments appeared after 90 hours. In solid media of pH 7 and 6.6 there was not an elongation of bacteria. In liquid media of pH 7 and 6.6 very long filaments were present 48 hours after *S. marcescens* had been inoculated. In media with lower sodium perborate content the bacteria were normal. Also in media containing boric acid, filaments were present. In culture media containing 305 mg. % of boric acid (containing the same quantity of boron of 250 mg. of sodium tetraborate) the growth was slow, but only 24 hours after *S. marcescens* had been inoculated a great number of filaments was present, many of them with vacuoles. Filaments were present especially in the liquid media. In media containing lower quantities of boric acid the cells were normal.

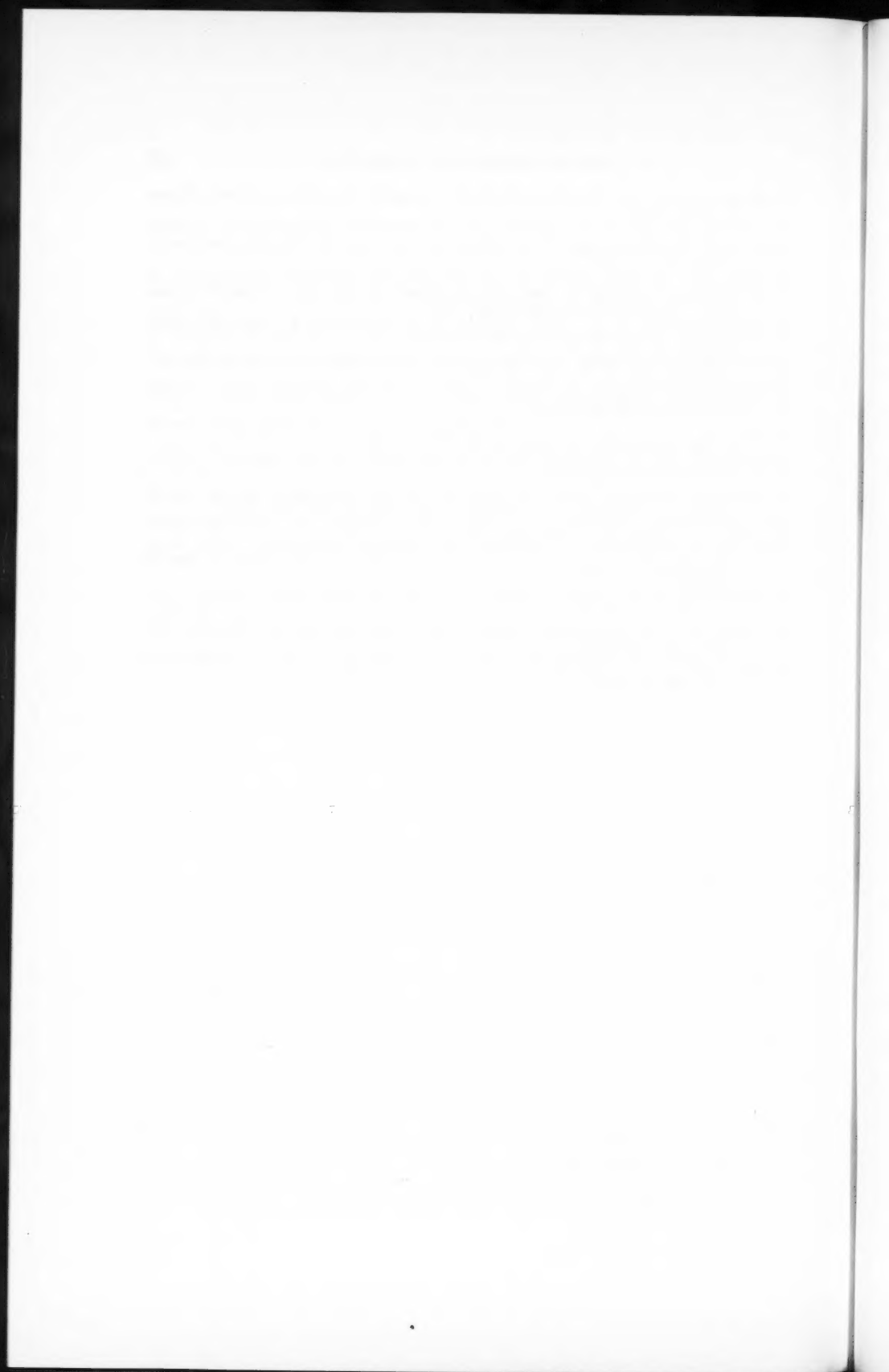
We tried other elements of the same group as boron in the periodic system to see if they could cause the appearance of filaments. We have prepared culture media containing aluminum sulphate and aluminum nitrate (in amounts such as to furnish aluminum in the same quantities as boron contained in the above-mentioned media). Thallium chlorate, thallium nitrate, and gallium oxide have been added to media in much lower quantities (5, 10, 25 mg.) on account of their toxic action. In all these culture media *S. marcescens* appeared normal.

The production of filaments in cultures of *S. marcescens* is a characteristic of boron and is not related to the compound of this element which is added to the culture media. The filaments were more evident at alkaline pH in solid culture media, while at neutral or acid pH they were much more evident in liquid media. The modification was not hereditary.

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## MORPHOLOGICAL STUDIES ON NODULE BACTERIA CULTURES (RHIZOBIUM SP.)<sup>1</sup>

ANNA LEHNER AND W. NOWAK

### Abstract

The evaluation of the purity and quality of nodule bacteria cultures, used for the inoculation of seeds, requires a useful morphological approach for control purposes. For that reason 23 sources of nodule bacteria were characterized with regard to their shape and form by means of living preparations. The typical stages of the nodule bacteria of various Leguminosae, appearing exclusively in the form of rods, were photomicrographed. These studies included bacteria isolated many years ago as well as those obtained in 1955. The inoculation material at hand consisted of slightly motile rods of straight and sometimes slightly bent form, the length of which differed even among nodule bacteria of the same species.

In relation to the systematic classification of the Leguminosae, the nodule bacteria variations isolated from the former were united in the following 10 groups of forms:

1. The forms from the *Lupinus* group as a morphologically homogeneous series.
2. The nodule bacteria of *Medicago sativa* L., *M. lupulina* L., and *Melilotus albus* Med. with transitional characteristics between the individual species.
3. The nodule bacteria of the *Trifolium* species, sometimes non-uniform, showed thick rods of variable lengths.
4. The group of the nodule bacteria of *Anthyllis vulneraria* L., *Lotus uliginosus* L., and *L. corniculatus* L. characterized as short rods. A typical feature seems to be the development of star-shaped accumulations.
5. The independent group of the nodule bacteria of *Onobrychis sativa* Lmk.
6. The independent group of *Ornithopus sativus* Brot.
7. The nodule bacteria of *Vicia faba* L. and *V. sativa* L. showing short and long rods with heavy mucus development.
8. The nodule bacteria of *Lens esculenta* Moench showing short rods of varying thickness.
9. The typical straight rods of *Pisum sativum* L. and *P. arvense* L.
10. The long rods of *Phaseolus vulgaris* L. and *P. nanus* L. differing from those of *Pisum* sp. only by the slight bend of the rods and a typical development of star forms.

The morphological differences we found appeared with such regularity in certain species that their occurrence was recorded in typical pictures.

### Introduction

Seed inoculation of the important agricultural group Leguminosae has been successful for decades. Numerous experiments have proved that the inoculation caused considerable yield increases in root and leaf. These observations and findings were described by Hiltner (10, 11, 12), Kronberger, (15), Lehner (16, 17, 18, 19), Stapp (34), Gisiger (7), and many others.

Good results require the use of tested nodule bacteria cultures for seed inoculation. The first usable cultures were handed to the farmers in 1904 by the Bayerische Landesanstalt für Pflanzenbau und Pflanzenschutz, on the initiative of Hiltner. Seed inoculation meant additional work to the farmer

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and was a completely new agricultural process, yet it succeeded within a short time because of the proved material gain.

At the same time the United States of America planned to produce a vaccine of nodule bacteria cultures for Leguminosae inoculation (8). Yet these experiments had no practical results. In the meantime the United States has taken up large-scale production of nodule bacteria cultures; widely divergent estimates indicated the annual production and distribution of enormous quantities of nodule bacteria cultures. A similar success of the seed inoculation was recorded in the countries of Northern Europe. Virtanen's researches had a big share in this development (40).

Various views on the biology of these microorganisms have been advocated in the technical literature. Many articles aimed at the recognition of morphological characters. These morphological data, frequently interspersed and mostly incomplete, will be listed in the following review, which is only a partial report owing to the popularity of the subject.

### Record of Literature

Statements on the morphology of the nodule bacteria (*Rhizobium* sp.) differ widely. This may be caused mainly by the selection of the samples examined. Apart from the agricultural utilization, the problem of nodule development in the Leguminosae is an interesting subject. Small wonder that many authors studied in detail the form, shape, and function of the nodules and their bacteria. Independent of each other, they gathered abundant material.

The data are often incomplete, however. Frequently it is not recorded whether the preparation came from an old or a fresh culture. The type of culture medium and the environmental conditions are often neglected.

The collective work of Burrell and Hansen (5), comprising the whole field and giving data from 1687 to 1916, and those of Löhnis (22, 23) reaching up to 1935, should be referred to especially. Data on morphology are interspersed therein. They will be condensed in this article.

Our special interest concerned the length of the rods. Almost all authors studied this morphological characteristic. Matzschita (25) described major rods (1–4  $\mu$ ) and minor swimmers. These rods are said to be of irregular and frequently humpbacked shapes. Fork-shaped or three-armed forms are found, too.

The latter forms have various names. They are called "involution forms" by Migula (26). Hiltner (10) described these forms for the first time. He found delicate, branched and globular so-called bacteroids in the nodules of *Ornithopus* and *Trifolium*, the development of which he ascribed to the influence of liquid, slightly acid media. Rods developed only on solid culture media, in his view. Zipfel (43) characterized the bacteroids as forms with special biological performances. The development of the bacteroids in the nodules was described by Štefan (37), Němec (28), and lately by Thornton (39). Müller and Stapp (27) considered the bacteroids as teratological forms.

According to Schanderl (31), they were habitat modifications of symbiotic bacteria of the Leguminosae which found their residence in the root nodules.

Apart from these main forms, bacteroids and rods, the literature on the subject records motile swimmers (up to a length of  $0.9\ \mu$ ) as well as large immotile cocci (according to Bewley and Hutchinson (3)). These forms were photomicrographed by Carroll (6). These shapes are ascribed by many authors to a modification of the culture media, e.g. there is the development of some teratological forms on agar cultures after the addition of CsCl and LiCl (27). Other biologists declare that the occurrence of rods is more consistent in the presence of certain carbohydrates. If these nutrients are used up during the culture period, if the rods are bred in mineral media ( $\text{CaPO}_4$ ,  $\text{MgCO}_3$ ) or under anaerobic conditions, swimmers and especially coccus-like forms are said to develop (3).

The cycle stated by the latter authors is illustrated by Rokitzka (30). This fact seems important, because the authors Bewley and Hutchinson (3) are not mentioned in this article and because their publications are not easily accessible in our country. An analogous life cycle of the nodule bacteria, containing preswarmers as well as motile and non-motile rods in addition to coccoid forms and swimmers, was described and outlined shortly by Waksman (41). Besides, Israelsky and Leonowitsch (14) referred to so-called R- and S-forms.

All these statements indicate that these forms are due to the prevailing culture conditions (33). Carroll (6) described and photographed rods in the nodule bacteria of cowpea (= *Vigna sinensis* = *Phaseolus riccardianus* Hort.), after a 48-hour cultivation period. He also showed bacteroids after a cultivation period of 8 days and coccoid phases after a period of 17 days, according to his statements. These, however, should be subject to review.

Müller and Stapp (27) found lively motile swimmers in young cultures, while short or longer rods prevailed in older cultures. Lochhead (20, 21) is of the same opinion, characterizing the nodule bacteria either as small round corpuscles or as straight, rod-shaped forms, according to the environmental conditions; Y- and T-forms may also be found. Baldwin and Fred (1), and lately Bergey's Manual (2), give a similar characteristic of the nodule bacteria comprised under the denomination *Rhizobium* sp.

Some essential points for the morphological distinction of certain nodule bacteria are found with Bisset and Hale (4), who made electron microscope preparations. The distinctive marks are based on the ability of certain species to develop coccoid swimmers with one, rarely two flagella, which become disengaged after the decomposition of the so-called "barred" form; they copulate and grow to small vegetative rods with one to three flagella. These phases are able to penetrate into the tissue of the root, by way of the root hair. The barred forms themselves are multicellular, considerably larger than the vegetative forms, and contain darkly stained aggregations at the cell wall. The nodule bacteria of lupins, sweet peas, and vetches are able to develop swimmers. Yet the nodule bacteria of cultivated peas, beans,

and clovers lack this ability. These plants contain bacteria, mostly unicellular, small motile rods; there are also a few intermediary forms.

The bacteroids in the nodules themselves have special forms, which, however, were not described by the above-mentioned authors.

More than 50 years ago Hiltner and Störmer (11) used the formation of the bacteroids in dextrose solutions for the distinction of two groups of bacteria from the Leguminosae. They contrast the group from *Lupinus*, *Ornithopus*, *Glycine*, and *Genista* (having rod-shaped bacteroids) to the group from *Pisum*, *Lathyrus*, *Vicia*, *Phaseolus*, *Medicago sativa*, *Trifolium*, *Anthyllis vulneraria*, *Onobrychis*, and *Robinia* (which have broad bacteroids with sprouts, and grow on gelatin in culture media). Bisset and Hale's (4) subdivision on the basis of the ability to produce swimmers is certified by the aforesaid apart from some exceptions.

The search for distinctive characters within the nodule bacteria was carried out by Hiltner and Störmer (11) for practical reasons, considering that the success of the inoculation must be dependent on the nature of the inoculation material. The two authors were completely uncertain of the morphology of the nodule bacteria. They ascribed the variability of the forms to an infection of their strains.

Almost all authors avoid the problem of the motility of the rods. Without doubt it depends on the development of mucilage, and Schneider (32) is the only author to describe in detail the immotility of *Rhizobium mutabile*.

The problems presented in the literature have induced us to observe our strains under comparable conditions on the same artificial nutrient medium.

### Test Methods and Test Material

Morphological studies were carried out on 23 strains of nodule bacteria, which were isolated from the Leguminosae listed in Table I. As may be seen, some of the strains belong to cultures gained already in the period from 1935 to 1954; yet there were also new cultures gained in 1955. The latter were isolated from Leguminosae cultivated and inoculated for this test on our proving grounds at Nederling near Munich and Brandhof (Mittelfranken). Older strains, which had been transplanted anew and examined morphologically, were used for inoculation.

A repeated microscopic examination of the samples listed in Table I was necessary in order to verify the results recorded in 1954 and 1955. Besides, the working schedule enabled us to look for correlations between the habitat of the Leguminosae and the morphology of the bacteria isolated\*.

It seemed necessary to know the morphology of nodule bacteria in comparable cultivation conditions as a starting point for our studies. Therefore all cultures were grown, on mannitol agar on one hand and in a mannitol

\* The soils of the above-mentioned proving grounds may be characterized in the following way (according to Schaeffler): Nederling: Flat, low terrace gravelly soils (gravelly, clayey sand) from a region rich in precipitation. The proving grounds Brandhof (near Neustadt/Aisch) have clayey Keuper sand soils deficient of lime and phosphoric acid, with adequate supply of potassium.



nutrient solution on the other, at normal room temperature. The cultures were examined at intervals of 1, 2, 4, and 6 weeks after the transplantation. In this way the influence of the age of the culture on rod morphology was investigated. A longer period of observation seemed of no practical use.

These morphological studies require frequent examinations, whether we intend to study pure cultures for scientific purposes or usable cultures for the inoculation of Leguminosae of agricultural importance. The latter cultures are those, dashed only slightly with cocci or rods of other species, used exclusively for seed inoculation and not for breeding as stock cultures.

The individual strains are isolated annually from fresh material in exacting, petty work. All purified and well-growing strains were preserved to allow morphological comparisons of newly won material with old cultures. There exist stained permanent microscopic slides, which were prepared by Kronberger (15) almost 50 years ago and they are still well preserved. These preparations correspond well with our present morphological findings.

Our own studies are concerned with pure and usable cultures of nodule bacteria of the most-cultivated Leguminosae. Microscopic tests are carried out on a large scale, especially in spring, and require an exact knowledge of the morphological qualities of this group of organisms. Yet this research is influenced to a large extent by the available optical facilities. During the last few years control has been carried out almost exclusively with the aid of the phase-contrast microscope. The examination of nodule bacteria cultures by means of the bright-field microscope was possible only to a limited extent, as only special stains gave a certain insight into the morphology. The phase-contrast microscope on the other hand permits the direct observation of living preparations, and eliminates the complications of staining. (See the comparative studies of Winkler, Knoch, and König (42).)

The results obtained by English and American scientists with the aid of the electron microscope cannot be taken into consideration, for lack of comparative objects. They show the problems still existing in this field. Above all, the development phases and the ability to form swarmers could not be observed with our optical facilities.

*We place the practical interests of the inoculation of Leguminosae into the foreground of our considerations*, as we have been concerned for years with the production of living Leguminosae vaccine. It must not be forgotten that some soils may be used for Leguminosae cultivation only after inoculation with suitable nodule bacteria. Yet the examination method based on morphological characters is only *one* resource, and a series of other physiological controls must also be applied.

The nodule bacteria are more or less uniform rods of various length. As a rule, the rods are straight; some are slightly bent. Differences in length were found not only in the nodule bacteria of various species of Leguminosae, but also within one "species" of nodule bacteria. Lüdecke and Poschenrieder (24) carried out some inoculation experiments on Leguminosae in order to clear up that problem.



Intermediary forms and development stages, including the bacteroids and the swarmers of some species, appear in ordinary cultures. They occur mostly in the forms living directly in the nodules and disappear during repeated transplantation on artificial nutrient media.

Bergey's Manual distinguishes six species of *Rhizobia* taking into consideration the flagellation, the shape of the bacteroids, certain morphological statements, and the biochemical reactions. According to the practical experiences gathered in "inoculation", even more subdivisions might be put up. Stapp lists nine groups for German agriculture.

The morphology of the rods in cultures has not found much consideration up to now. Most of the authors studied the manifold shapes of the bacteroids. For a change it was interesting to study the shapes of the most important nodule bacteria under *constant cultivation conditions* and to show the differences in pictures.

Different individual data in the literature (2, 13, 25) state variations of 1-4  $\mu$ , 1-3  $\mu$ , and 1.2-3  $\mu$  of length for the nodule bacteria of the same species. On the contrary, Löhnis (22, p. 590) characterized the nodule bacteria as short rods (1-2  $\mu$ ). Longer rods up to 3  $\mu$  occur rarely according to him. All these statements must not be taken unconditionally, however.

The data in the literature did not always show clearly the developmental stage of the strains examined or describe the nutrient medium. We tried to find the length of the rods under similar cultivation conditions, and the changes with age of the strains examined.

### Morphological Studies

Our observations are based on comprehensive picture material and the illustrations (Figs. 1-42) represent a selection thereof. Only rich material permits comparative studies in various directions. Thus we tried to find correlations between the rod morphology, the age of the culture, and the habitat of the Leguminosae, as far as the cultivation conditions remained constant. In special individual cases we also tried to find differences in the rod morphology on different cultivation soils.

The nodule bacteria cultures were arranged according to botanical systematic associations of their symbionts (Table I). We used the statements of Garcke in this case. This enables us to group the nodule bacteria according to the congenial correlation of the Leguminosae, to describe them individually, and to compare them. The division of the nodule bacteria on the basis of their ability to form swarmers according to Bisset and Hale (4) or on the basis of the shapes of their bacteroids according to Hiltner and Störmer (11), indicated at the beginning of our article, has been neglected in this case. Without intending to detract from the findings of the above-mentioned authors, we shall try to study the shape of the nodule bacteria in culture considering the circumstances of the experiment.

The opportunity for such a synopsis presents itself in the group of the nodule bacteria isolated from *Lupinus* sp. These are rods of an average

length of 2.5–3  $\mu$ , slightly bent, sometimes even comma-shaped owing to their slightly thickened poles. For that reason the rods are convex in some positions in the preparation, which causes certain reflections of light, and the rods seem hollow.

Our studies on the nodule bacteria from the four different stocks of *Lupinus* showed no remarkable differences, neither with regard to the age nor to the habitat. In detail, there were no variations in the nodule bacteria of all four *Lupinus* species, neither from the different Bavarian habitats nor in the comparison of fresh and 6-week-old cultures (Figs. 1–5). Only the nodule bacteria of *Lupinus albus* L. cultivated in the proving grounds at Brandhof (see Table I) showed typical symptoms of age after 4 weeks. The aging of the culture was shown by more intensive mucus development and the rods became more contrasting, globular, and sometimes flat in form (Fig. 6). The cultures were grown on mannitol, as well as on a special *Lupinus* agar. Using the latter, the thickness of the rods was markedly greater after a cultivation period of 1 week than it was in the forms grown exclusively on mannitol agar (Figs. 7 and 8). Probably this is due to the more favorable nutrient conditions on the *Lupinus* agar; this observation was recently made by Stapp and Knösel (35, 36) in their studies on *Agrobacterium* using substrates of different composition.

Nevertheless, we can confirm that the nodule bacteria united in Bergey's Manual under the species *Rhizobium lupini* (Schroeter) are a morphologically homogeneous group of forms, with the exception of the nodule bacteria of *Ornithopus sativus* L. The latter had been classed with this group of forms (2), because of its similar physiological behavior. Because of the bacteroid forms to be found, Hiltner and Störmer (11) classed the rhizobia of *Ornithopus* with the *Lupinus* group of forms.

The nodule bacteria of *Medicago sativa* L. and *M. lupulina* L. have rods of different length. The short rods from *Medicago sativa* L. and *M. lupulina* L. are filled with slime in most cases and therefore appear broader than usual under the microscope. The similarity of the short rods of the rhizobia from *M. sativa* L. and those from *M. lupulina* L. is very clear and demonstrates their correlation (Figs. 9 and 10). The longer rods of the nodule bacteria of

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Apart from the kind of the nodule bacteria shown, the year of their isolation and the age of the culture is given. The denomination "B" or "N" instead of the year means that the cultures were isolated in 1955 from the Leguminosae cultivated on the proving grounds (Brandhof = B or Nederling = N). All pictures are photomicrographs (Leica) which were taken with the phase-contrast microscope and enlarged to 1100 $\times$ .

FIG. 1.	Nodule bacteria isolated from	<i>Lupinus albus</i> L.	1954, 2 weeks.
" 2.	"	"	"
" 3.	"	"	"
" 4.	"	"	"
" 5.	"	"	"
" 6.	"	"	"
" 7.	"	"	"
" 8.	"	"	"
" 9.	"	"	"
		<i>L. luteus</i> L.	1954, 2 "
		<i>L. angustifolius</i>	1949, 2 "
		<i>L. perennis</i> L.	1949, 2 "
		<i>L. angustifolius</i>	1949, 6 "
		<i>L. albus</i> L.	B, 4 "
		<i>L. angustifolius</i>	1949, 2 "
		<i>L. perennis</i> L.	1949, 1 week
		<i>Medicago sativa</i> L.	1948, 4 weeks

PLATE I

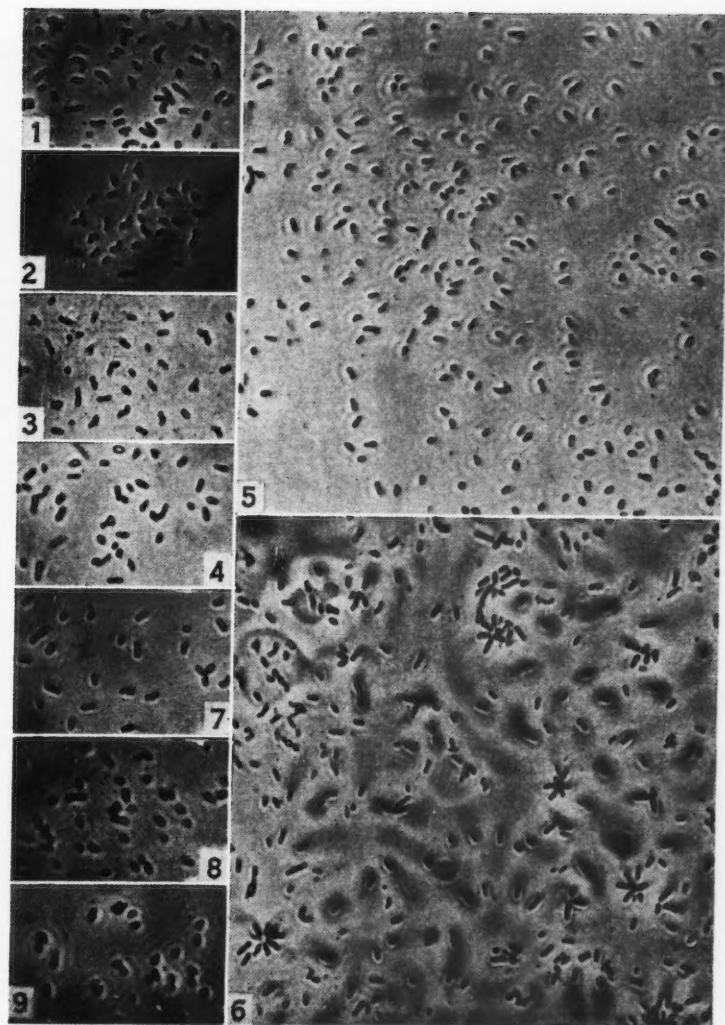


PLATE II

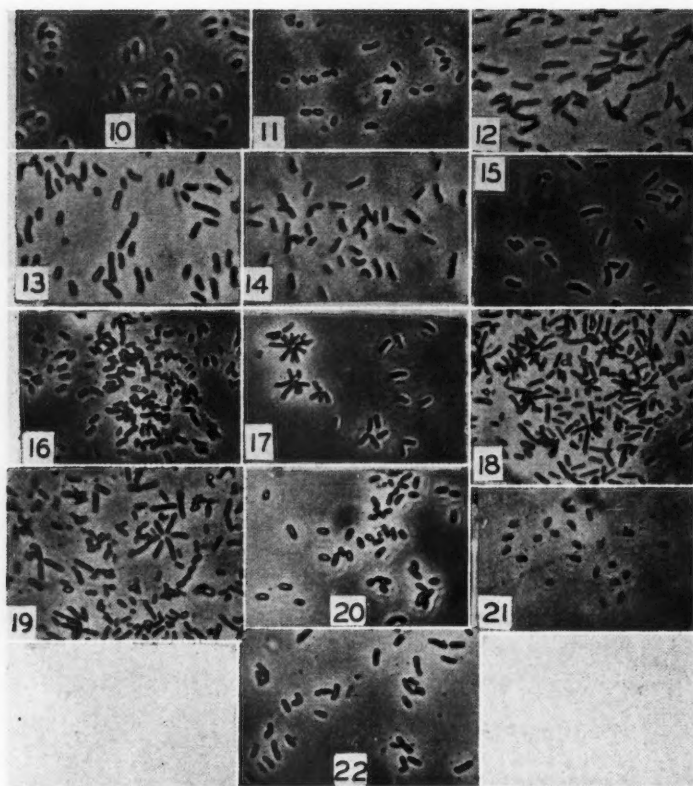


FIG. 10.	Nodulē bacteria isolated from	<i>M. lupulina</i> L.	1935, 4 weeks
" 11.	" " " "	<i>Melilotus albus</i> Med.	B, 4 "
" 12.	" " " "	<i>Medicago sativa</i> L.	1948.
" 13.	" " " "	<i>Melilotus albus</i> Med.	1950.
" 14.	" " " "	<i>Trifolium pratense</i> L.	1946, 3 days
" 15.	" " " "	<i>T. repens</i> L.	1946, 1 week
" 16.	" " " "	<i>T. hybridum</i> L.	1950, 4 weeks
" 17.	" " " "	<i>T. incarnatum</i> L.	1950, 2 "
" 18.	" " " "	<i>T. incarnatum</i> L.	B, 4 "
" 19.	" " " "	<i>T. incarnatum</i> L.	B, 4 "
" 20.	" " " "	<i>Anthyllis vulneraria</i> L.	1948, 2 "
" 21.	" " " "	<i>Lotus uliginosus</i> L.	1948, 4 "
" 22.	" " " "	<i>L. corniculatus</i> L.	1948, 2 "

PLATE III

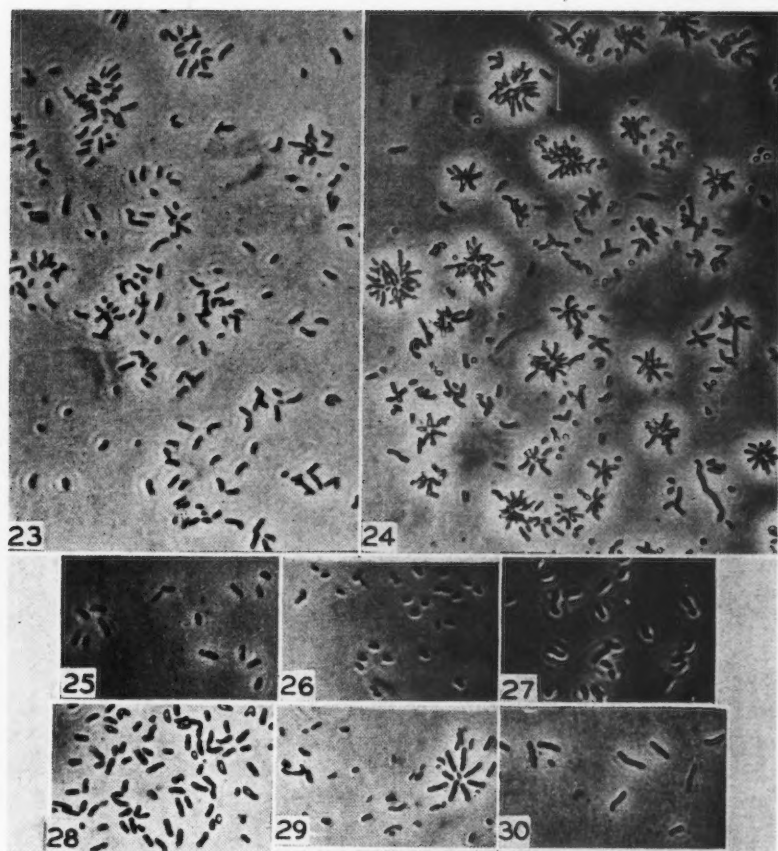
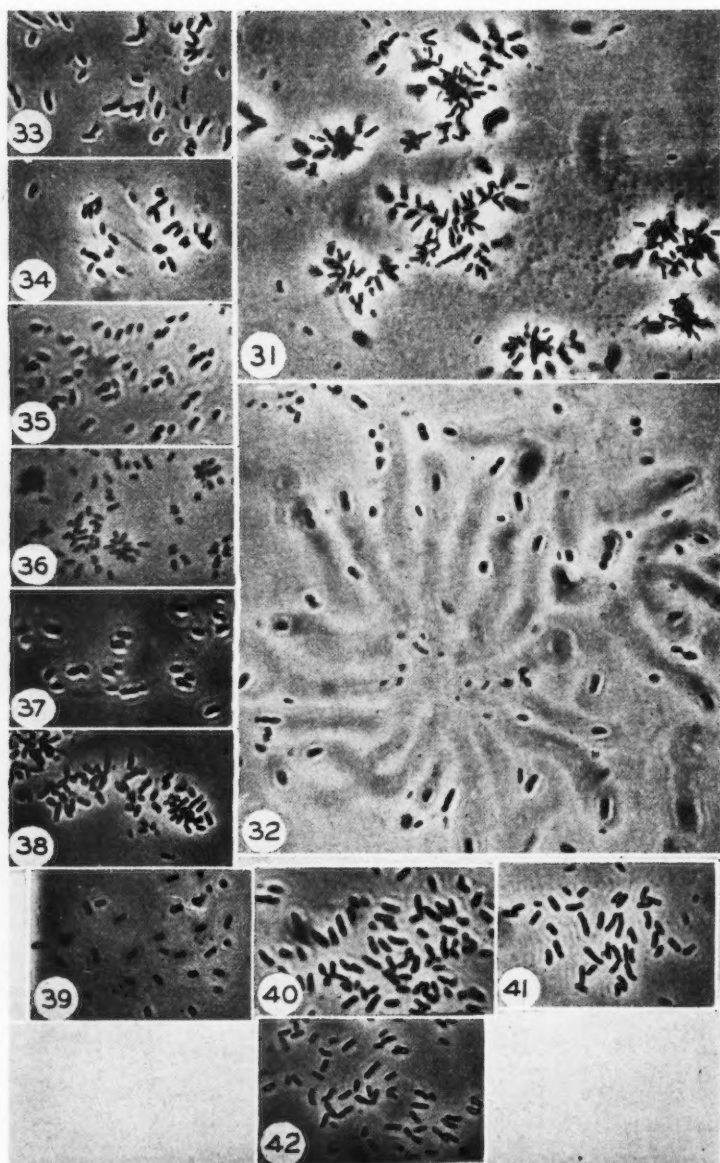


FIG. 23.	Nodule bacteria isolated from	<i>Anthyllis vulneraria</i> L.	1948, 6 weeks
" 24.	" " " "	" <i>Lotus corniculatus</i> L.	1948, 6 "
" 25.	" " " "	" <i>Onobrychis sativa</i> Lmk.	1946, 1 week
" 26.	" " " "	" <i>O. sativa</i> Lmk.	N, 1 "
" 27.	" " " "	" <i>O. sativa</i> Lmk.	1946, 4 weeks
" 28.	" " " "	" <i>Ornithopus sativus</i> Brot.	N, 2 "
" 29.	" " " "	" <i>O. sativus</i> Brot.	B, 4 "
" 30.	" " " "	" <i>O. sativus</i> Brot.	1949, 6 "

PLATE IV





*M. sativa* L., found in the stems cultivated already in 1948, show a marked similarity to those of *Melilotus albus* Med. This similarity is still more obvious, if these bacteria were taken from strains cultivated on agar for a long time (Figs. 12 and 13). In the *Melilotus* rhizobia isolated in 1955, shorter forms prevail, so that we may assume a correlation with the time of isolation from the nodule (Fig. 11). As this assumption is not founded experimentally, we may unite the rhizobia of *Medicago sativa* L., *M. lupulina* L., and *Melilotus albus* Med. under another group of forms (Figs. 9-13). The collection of these three sources of nodule bacteria under the denomination *Rhizobium meliloti* Dangeard, found in Bergey's Manual, can be confirmed by our department. It should be mentioned further that only short rods were found in the rhizobia of *Medicago lupulina* L. This observation was confirmed repeatedly and is obvious also in the figure.

Now let us turn to the rhizobia from the *Trifolium* species. A combination of the nodule bacteria from the four different species of *Trifolium* might suggest itself, yet it is justified only with the nodule bacteria of *Trifolium pratense* L. and *T. repens* L., from the morphological point of view. Here we have longer and stronger rods, also to be seen in Kronberger's 50-year-old preparations. The nodule bacteria of *Trifolium hybridum* L. and *T. incarnatum* L. mostly show short rods covered with slime, looking very thick. They are often bent, appearing hollow under the phase-contrast microscope (Figs. 14-17). But there are also longer rods in the latter group (Figs. 18 and 19). A typical feature of this group of forms, called *Rhizobium trifolii* in Bergey's Manual, is the ability to form little stars, described by Stapp and Knösel (36) and observed by our department especially in the nodule bacteria of *Trifolium incarnatum* L. There are no correlations between the age of the cultures and the forms.

The nodule bacteria of *Anthyllis vulneraria* L., *Lotus uliginosus* L., and *L. corniculatus* L. make up a morphologically homogeneous group of forms (Figs. 20-22). There are mostly short rods about  $2\mu$  long, sometimes covered with slime. A typical feature is the ability to form little stars, indicated before in our article (29). There are no correlations to the age of the culture, up to 4 weeks. Older cultures with longer rods are shown in Figs. 23 and 24.

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FIG. 31.	Nodule bacteria isolated from	<i>O. sativus</i> Brot.	N,	6 weeks
" 32.	"	"	B,	4 "
" 33.	"	"	"	"
" 34.	"	"	"	"
" 35.	"	"	"	"
" 36.	"	"	"	"
" 37.	"	"	"	"
" 38.	"	"	"	"
" 39.	"	"	"	"
" 40.	"	"	"	"
" 41.	"	"	"	"
" 42.	"	"	"	"

---

The nodule bacteria of *Onobrychis sativa* Lmk. and *Ornithopus sativus* Brot. were seen as an independent group of forms. The former, whose R and S forms have been studied by Israilsky and Leonowitsch (14), show delicate short rods as well as variously bent and somewhat longer rods, and older cultures are filled with slime. One might speak of habitat varieties in this case (Figs. 25, 26, and 27).

The nodule bacteria of *Ornithopus sativus* Brot. have short and sometimes slightly bent and Y-shaped rod forms. They are similar to those of the *Lupinus* group, especially their physiological attributes (2). The repeated occurrence of longer and sometimes thinner rods, often forming star-shaped conglomerations (prevailing in older cultures), requires the setting-up of a special group of forms (Figs. 28-30). Old age symptoms with slime "cakes" after 4 to 6 weeks are shown in Figs. 31 and 32.

The typical feature of the nodule bacteria isolated from *Vicia faba* L. and *V. sativa* L. is a heavy development of mucus. The former have somewhat longer rods, while the short rhizobia of *V. sativa* L. project from the mucus-like dots, appearing shorter than they are in the photomicrograph (Figs. 33 and 34).

Special attention must be paid to the nodule bacteria of *Lens esculenta* Moench. There are thick, short rods embedded in slime and more delicate rods. Both forms have the ability to develop star-shaped formations (Figs. 35 and 36).

Characteristic forms are shown by the nodule bacteria of *Pisum sativum* L. and *P. arvense* L. The bacteria of *P. sativum* L., in many cases filled with slime, show longer and straight rods of about 3.5  $\mu$  length, especially in the strains isolated in 1955. Their form is always typical and justifies the setting-up of an individual group of forms (Figs. 37-40). These findings differ to a certain extent from earlier data in the literature, according to which shorter rods of an average length of 2  $\mu$  are typical for these forms (38).

The 10th group of forms comprises the nodule bacteria of *Phaseolus vulgaris* L. and *P. nanus* L. Their rods are long, up to 3.5  $\mu$  of length. Unlike the *Rhizobium* of *Pisum sativum* L., these rods are mostly bent, which is also obvious in rods embedded in slime. A remarkable fact is that little stars and star-shaped conglomerations are formed of the longer rods, showing granular inclusions moreover. This is typical in the nodule bacteria of *Phaseolus nanus* L. (Figs. 41 and 42). There are no differences concerning age and habitat.

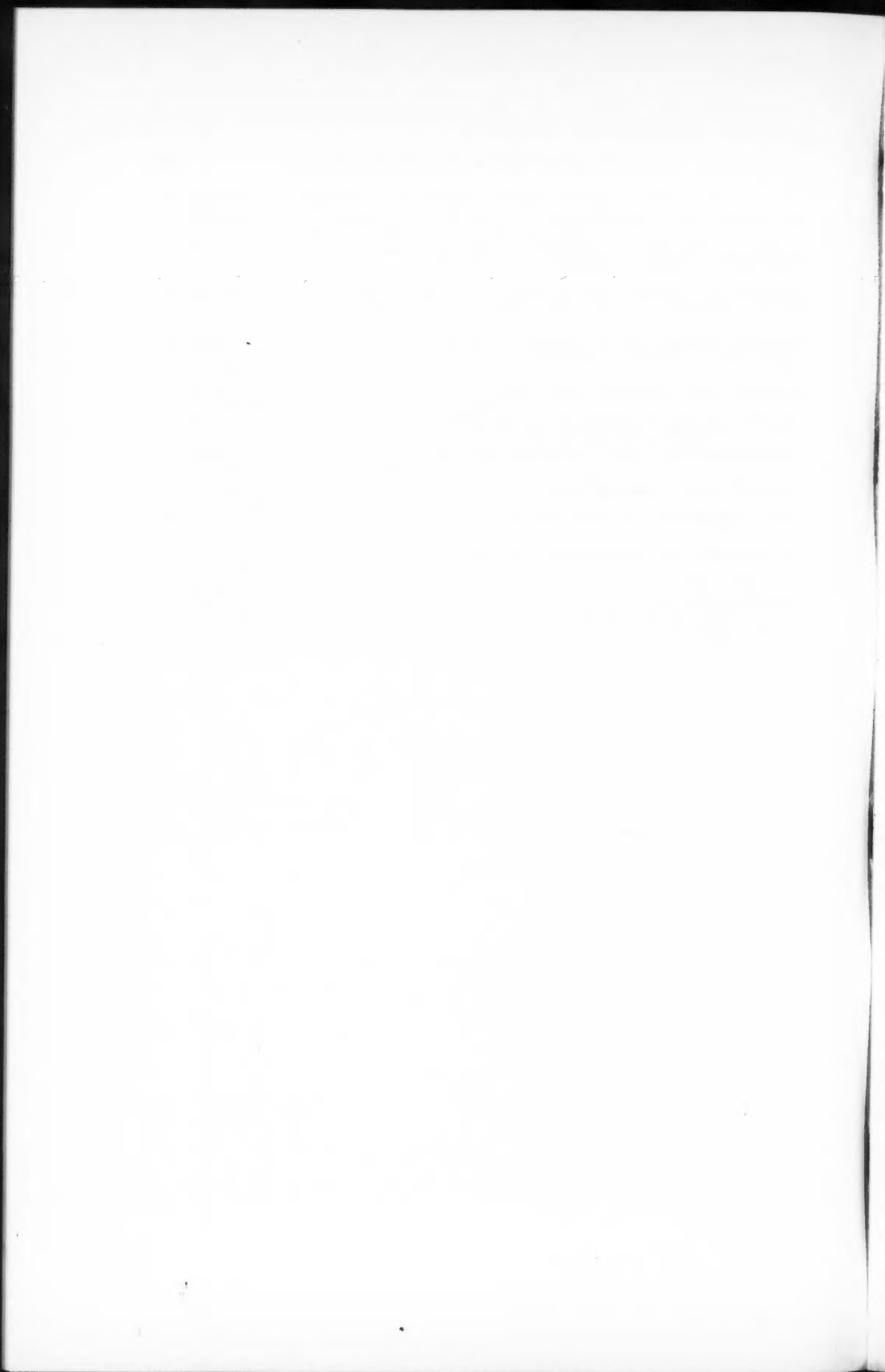
By the comparison of various forms of nodule bacteria, 10 groups were recognized from the 23 sources of nodule bacteria examined. Thereby we showed the range of dispersion of the rod length within these groups of bacteria and tried to define it. We further sought to review the general data on the lengths of the rhizobia widely dispersed in the literature. In our opinion such a review and evaluation of morphological notions, especially the "length of the rods", may facilitate the evaluation of ordinary cultures of nodule bacteria.

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